Pigs as animal model for low-birth-weight babies

Developing cognitive tests and examining neuroprotection

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Pigs as animal model for low-birth-weight babies

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Varkens als model voor baby's met een laag geboortegewicht
De ontwikkeling van cognitieve tests en onderzoek naar neuroprotectie

(met een samenvatting in het Nederlands)

Proefschrift

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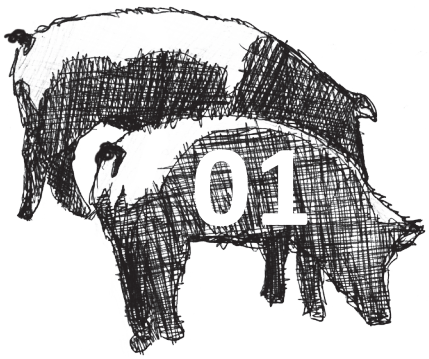

General introduction

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In this thesis I study the cognitive performance of piglets with low-birth-weight caused by intra-uterine growth restriction (IUGR), and assess the effects of a possible therapy to prevent IUGR-related brain damage and associated cognitive impairments. To achieve these goals, several conditions had to be fulfilled. First, an appropriate cognitive task was selected and validated. Then, the effects of IUGR in this task were investigated using this task. In addition, basic pharmacokinetic data of the putative therapeutic drug were collected in sows and piglets. Finally, the effects of the therapeutic drug on IUGR piglets were assessed. The above-mentioned topics will be introduced in this chapter, and an outline of the thesis will be presented.

The pig in research

The domestic pig *(Sus scrofa domestica)*, hereafter called 'pig', has lived in close proximity to humans since ancient times and has been used as a species in research for many reasons. There are two main fields of research in which the pig is currently used as (model) species, namely animal welfare research and biomedical research. In the field of animal welfare, the commercially kept pig is studied to be able to improve its quality of life when kept in husbandry systems not adapted to the behavioural needs of the species, but optimised to reach high production goals. In biomedical research, the pig is applied to produce information applicable to human health and disease.

The pig in welfare research

In the Netherlands, in 2011 about 12 million pigs were kept for meat production purposes on around 6500 farms. The Dutch pig producing sector is very efficient and, together with Denmark, reaches the highest piglet productivity in the world (LEI. 2012b). In these highly efficient systems, animal welfare relies strongly on husbandry conditions (de Greef et al. 2011). For example, animals should be able to cope with limited space, mixing of groups, no bedding material and automated feeding systems. This is not always in consensus with their behavioural needs, such as establishing a stable hierarchy, foraging and exploration (Stolba and Wood-Gush. 1981). In conventionally kept pigs, abnormal behaviours such as tail biting and belly nosing are frequently observed and are considered as indicators of reduced welfare (Deen. 2010, Statham et al. 2011). The welfare of animals kept for production purposes has received increased attention in recent years. On the one hand, the market asks for livestock products that are produced adhering to higher welfare standards, on the other hand the costs involved with enhancing welfare will raise prices. Solutions to ensure adequate welfare levels are expected by society from politics but also from the industry itself, stakeholders and science (de Greef et al. 2011). The welfare of pigs can be assessed in a practical way, for example by comparing welfare elements between different husbandry systems (Cagienard et al. 2005). By a more fundamental approach, pigs' behavioural needs, physical, emotional and cognitive abilities and preferences can be studied. This can be done with specifically designed tests (e.g. preference (Špinka et al. 1998) or social isolation (Weary et al. 1997) tests) or by studying animals in their natural habitat (Stangel and Jensen. 1991, Stolba and Wood-Gush. 1981). However, the lack of specific scientific tools to evaluate welfare in pigs is one of

the major reasons for the difficulty to demonstrate adequate welfare in an objective way (Deen. 2010).

The pig in biomedical research

Although the use of the pig in animal research is relatively limited (in the Netherlands in 2010, pigs made up 1.83% of all animals used for animal experimentation (NVWA. 2011), interest in the pig and miniature pig (hereafter called 'minipig') as model species is growing. The scientific advantages of using pig models in biomedical research in general were first described in detail in the 1970s (Baldwin and Stephens. 1973, Chaput et al. 1973). In various areas of research, the pig is already an established model species, in particular owing to the anatomical similarities to humans (body size, cardiovascular system, skin, urinary system), functional similarities (gastrointestinal system, immune system) and the availability of disease models (diabetes, atherosclerosis, gastric ulcer, wound healing) (Gad. 2007, Swindle and Smith. 1998, Mortensen et al. 1998). Pigs are frequently used for the preclinical characterization of cardiovascular, diabetes and dermatology drugs. Furthermore, compared to mice, the immune system of the pig is more similar to that of humans, with 80% similarity on compared variables between pigs and humans versus 10% between mice and humans (Schook et al. 2005). The sequence and chromosome structural homology of the pig genome also shows strong similarity to that of humans (Chen et al. 2007, Groenen et al. 2012, Lunney. 2007, Petersen et al. 2009). Biotransformation of many drugs in man and pig is similar, as is shown by liver metabolism studies (Witkamp and Monshouwer. 1998). Pigs appear to be suited for most routes of drug administration and for the evaluation of most pharmacological, pharmacokinetic (absorption, distribution, metabolism and excretion; ADME) and toxicological endpoints. To reduce the amount of a drug needed for efficacy and safety testing, the minipig is often selected for preclinical drug studies. A short phylogenetic distance, i.e., high resemblance between the model species and the species to be modelled, is expected to increase the relevance and generalizability of results obtained in the model species (van der Staay et al. 2009). It has been suggested that (mini)pigs could serve as a substitute for dogs and nonhuman primates (Nunoya et al. 2007). Research on (mini)pigs may fill the gap between preclinical studies in rodents and clinical trials in humans (de Groot et al. 2005, Lind et al. 2007, Nunoya et al. 2007, Vodicka et al. 2005).

The pig in neurobehavioural research

Studying pig brains

The pig and miniature pig show multiple advantageous characteristics compared to rodent species that have led to an increase in the utilization of (mini) pigs in studies modelling neurobehavioral (dys)functions. The cerebral cortex of pigs, unlike that of mice or rats, has cerebral convolutions (gyri and sulci) similar to the human neocortex. This may facilitate physiological investigations and neuropathological comparisons with the human brain (Alisky. 2006). Its relatively large gyrencephalic brain makes the (mini)pig, particularly younger animals, well suited for (non-invasive) imaging techniques (Arnfred et al. 2004, Danielsen et al. 2001). Imaging research has increasingly been conducted in recent

years as a technique to further investigate pigs as model animals (particularly developmental models).

With their relatively large, gyrencephalic brain, and pharmacokinetic characteristics highly similar to humans, pigs can be the species of choice as an intermediate between rodents and humans. Additionally, the early developmental stages of the piglet brain closely parallel that of humans (Duhaime et al. 2000). A number of initiatives are actively evaluating and promoting the pig as a model animal species (e.g. the National Swine Resource and Research Center (NSRRC) and the EU Sixth Framework Program project RETHINK that evaluates the potential impact of toxicity testing in the minipig as an alternative approach in regulatory toxicity studies). Due to its smaller body size, the minipig is better suited to be housed and tested under laboratory conditions than the domestic pig. However, the potential of (mini)pig-based animal models for investigating (dys)functions of the nervous system and their consequences for the regulation of normal and abnormal behaviour is still in its infancy (Nielsen et al. 2009, Schook et al. 2005). A range of animal models based on different animal species is required for extrapolating and translating results from animal studies to humans (Roberts et al. 2003).

Cognitive studies in pigs

There is a wealth of tests for assessing learning and memory in rodents. However, these cannot be transferred one-to-one to other species. To use pigs in cognitive (bio)behavioural research, validated tests are urgently needed. An extended overview of the history, development and prerequisites of cognitive studies in pigs is given in chapter 2. To summarize, for a variety of reasons the pig is used in bio(behavioural) research to improve the welfare level of the species itself when kept in intensive husbandry systems or to serve human medicine. In many cases, the pig is preferred over rodents, or chosen as an intermediate species between rodents and humans in preclinical biomedical research. The number of studies in this biomedicine, and in research assessing pig welfare is steadily increasing, reflecting a growing appreciation of the pig as subject of scientific research. A lack of reliable and validated cognitive tests, however, retards the development of animal models with pigs as subjects and limits the quality of results obtained.

Low-birth-weight in humans and a piglet model

Low-birth-weight in humans

According to World Health Organization statistics, worldwide about 30 million low-birth-weight (LBW) babies are born annually, which is 23.8% of all births (WHO. 2012a). In more developed countries this percentage is lower (e.g. in 2002-2003 8% in the USA (Ergaz et al. 2005), but the number of children surviving, owing to improved pre-, peri-, and postnatal medical care, has rapidly increased over the last decades. The survivors, however, may suffer from the long-term consequences of early growth restriction. Quality of life of the survivors is causing more and more concern (Karimi et al. 2011).

Definitions

Low-birth-weight is not an ailment, but is generally seen as a read-out parameter for the underlying cause, namely intra uterine growth restriction or retardation (IUGR). A purely biological definition of IUGR refers to a failure of a foetus to reach its genetic growth potential (Cox and Marton. 2009, Ergaz et al. 2005, Klaric et al. 2012). Clinically, a child's birthweight that is lower than a predetermined cut off point is generally defined as having low-birth-weight (LBW) (Ergaz et al. 2005), but standards and definitions differ per study and hospital. Different cut off points are used: birth weight ≤2500, ≤2000, ≤1500 or ≤1000g (Breslau et al. 1996, Breslau and Chilcoat. 2000, Cox and Marton. 2009, Elgen et al. *in press*, Groen-Blokhuis et al. 2011, Hack. 2006, Johnson and Breslau. 2000, Li and Sung. 2008, McFarlin. 1994, Mu et al. 2008, Saigal et al. 1994), a sex-specific percentile (in general the 10th) for gestational age at birth (Bos et al. 2001, Gardosi. 2005, Geva et al. 2006, Scherjon et al. 1998, Theodore et al. 2009) or a weight ≤ a population mean minus 1, 1.5 or 2 times the standard deviation of this mean (Pihkala et al. 1989). Low-birth-weight children can be born full term or preterm. Unfortunately, not every study includes this variable in their specification of low-birth-weight. This makes it difficult to differentiate between consequences associated with being born preterm, IUGR, or a combination of both. A child born term but with a low weight can be called small for gestational age (SGA) or dysmature. The weight of a preterm neonate can be appropriate for its gestational age but its body immature, because of the moment of birth.

When a broad criterion to define LBW/IUGR is applied, it may be valid in large populations, but there is the risk that some of the infants included should not be included as growth restricted. They are included in the cohort while they just fall in the lower range of the normal distribution of the population (Bos et al. 2001, Cox and Marton. 2009, Ergaz et al. 2005). The same holds true when birth weight is used as the only measure for growth restriction; the reliance on only one criterion makes it difficult to discriminate between causes (Scherjon et al. 1998).

Causes

IUGR, which results in LBW, can have many underlying causes. All living creatures show plasticity during early (prenatal) development. To a certain extent they are able to adapt to the environment they are subjected to. These intra-uterine adaptations prepare them for the type of world into which they are born (Bateson et al. 2004, Smart. 1993). This means that the (aversive) intra uterine conditions the foetus is immersed in will influence its pre- and postnatal development. The birth weight of a baby is determined by its genetic growth potential but also by the ability of the mother to deliver oxygen and nutrients to the placenta and the placental ability to deliver these to the foetus (Cox and Marton. 2009). Its genetic growth potential will only be realised under optimal conditions. Maternal malnutrition or under-nutrition (Ergaz et al. 2005, Smart. 1993), maternal disease, low socioeconomic status (Cox and Marton. 2009), foetal chromosome abnormalities, (congenital) viral, bacterial and protozoal infections, adolescent pregnancy, maternal substance abuse, living at high altitude (lower oxygen mtension)

(Cox and Marton. 2009, Ergaz et al. 2005, Klaric et al. 2012), (pre)eclampsia, prematurity, multiple births, a short inter pregnancy interval, and thrombophilia (Ergaz et al. 2005) can all negatively influence the intra uterine growth of a foetus.

Irrespective of its underlying causes, insufficient placental functioning is generally seen as the main cause of growth restriction of a foetus (Cox and Marton. 2009, Ergaz et al. 2005, Klaric et al. 2012). While pure placental causes are rare (Cox and Marton. 2009), placental pathology is the main factor impairing growth of the foetus, but the abnormality in most cases is part of a genetic disorder of the conceptus or part of a maternal disorder (Cox and Marton. 2009). Although growth restriction is generally detected during the second or third trimester of pregnancy, time of onset and severity are highly variable. Consequently, the group of children born small for their gestational age is very heterogeneous (Bos et al. 2001).

Cognitive implications

Foetuses diagnosed as growth restricted are at a higher risk of intra uterine death and neonatal mortality. For surviving neonates, a number of serious consequences have been identified (Cox and Marton. 2009, McFarlin. 1994). Focussing on the cognitive implications only, it is known that IUGR can lead to a degree of failure of brain growth, and a lower brain weight (Cox and Marton. 2009) and volume (Martinussen et al. 2009). Later in life, poorer cognitive function, learning problems, difficulties with spatial orientation, and lower academic performance are found, compared to normal-birth-weight (NBW) controls (Chaudhari et al. 2004, Frisk et al. 2002, Hack. 2006, Ido et al. 1995, Kessenich. 2003, Leitner et al. 2005, Martinussen et al. 2009, O'Keeffe et al. 2003, Silva et al. 2006, Strauss. 2000), even though LBW children showed a normal intelligence and were free of neurological handicaps (Hack et al. 1992, Johnson and Breslau. 2000). LBW neonates are at increased risk to develop psychiatric disorders and behavioural problems in childhood and during young adulthood, ranging from, for example, attention problems (Breslau and Chilcoat. 2000, Elgen et al. *in press*, O'Keeffe et al. 2003, Shum et al. 2008), to depression (Raikkonen et al. 2008). The risk of development of such problems is related to the child's environmental conditions: in disadvantaged communities or countries LBW children are found to be at higher risk (Breslau and Chilcoat. 2000, Gardner et al. 2003).

Low-birth-weight in piglets

Pig breeders select for high fecundate sows to improve farm efficiency. This leads to increased litter sizes in commercial pig farming, in which increased numbers of piglets with a low-birth-weight can be found (Beaulieu et al. 2010, Milligan et al. 2002, Quiniou et al. 2002). This increase is not only seen in commercially farmed pigs, but also in minipig breeds kept for biomedical purposes or as a pet. One regularly observes piglets with a birth weight that is lower compared to the rest of their birth cohort (Ellegaard, pers comm. 2011, Myrie et al. 2011).

The LBW piglet as a model for humans and its own species

Although the reproductive system of pigs differs from that of humans (notably,

two bicornate horns, an epitheliochorial placenta, and a large number of offspring in sows versus a simplex uterus, a hemochorial placenta, and in general only one child per pregnancy in women), the main cause behind intra uterine growth restriction in piglets matches the cause of human IUGR, namely the adaptive response to poor perfusion to maintain pregnancy (Blomberg et al. 2010). Both species show a below-average birth weight in some neonates and both neonatal counterparts are likely to develop pre- peri- or postnatal complications. In pigs bred for meat production, the consequences of LBW have mostly been studied with respect to production and growth parameters (Gondret et al. 2005, Milligan et al. 2002, van der Lende and de Jager. 1991). Nothing is known about the neurological or cognitive consequences of LBW in piglets. The study of LBW in piglets could therefore not only benefit humans, but also the welfare of the species itself. Knowing whether LBW piglets are cognitively affected could aid postnatal care for these vulnerable animals. Eventual cognitive deficits could be taken into account when designing proper housing and management systems. Probably the results could even steer the public debate about high mortality rates in large litters. This discussion should involve the consequences on health and welfare for the surviving LBW piglets. As mentioned previously in 'cognitive studies in pigs', to be able to assess cognitive performance adequately, validated behavioural tests are urgently needed.

IUGR and/or low-birth-weight have been studied in other species, but most studies have induced the growth restriction experimentally (i.e. through partial ligation of uteroplacental vessels (Eixarch et al. 2012, Mallard et al. 2000), maternal protein restriction (Fernandez-Twinn et al. 2003, Mallard et al. 2000) or passive smoking (Huang et al. 2009). The advantage of the LBW piglet as a model species for humans could be that the mechanisms behind the restriction of growth are comparable and naturally induced. A disadvantage is that acquiring these pigs is less controlled and more uncertain. The latter because techniques to assess intra uterine growth in pigs such as ultrasound examinations are not yet fully developed and standardized, partly due to the unsolved problem to reliably indentify individual foetuses repeatedly during the course of pregnancy. If and how many LBW animals are born in a specific litter can only be observed after parturition.

Defining low-birth-weight in piglets

LBW is generally used as the main read-out parameter for IUGR after birth, and it is itself not a solo ailment. For pigs, currently no reliable measures exist to prenatally determine IUGR in individual piglets. This in contrast to human follow-up during pregnancy, where regular ultrasound examinations and fundal height measurements long before delivery yield reliable indications of whether a foetus is restricted in growth. For pigs almost no standards (e.g. femur length or head circumference) for intra uterine growth exist. Even with these standards present it would be difficult and expensive to follow-up on the same piglet during pregnancy, and to match this individual to the correct newborn after delivery. LBW therefore at the moment appears to be the best indicator for IUGR in piglets. But again, no standard measures are used across studies and absolute cut-off points are not defined. In a study by D'Inca et al. (2010), a piglet with a weight at least 1.5x the standard deviation (SD) below its *litter mean* was defined

as LBW, while Wise et al. (1997) classified a piglet as LBW when its birth weight was at least 1SD or 2SD's below the *population mean*. As birth weight in piglets is related to litter size (as it is in humans; twins are generally born with a lower birth weight compared to singletons (Naeye et al. 1966), LBW in the experiments outlined in this thesis is generally defined as follows:

LBW: All animals with a birth weight at least 1 SD below the average litter weight
The normal-birth-weight (NBW) control piglets derived from the same litter are determined as follows:

1) *All LBW animals are excluded from the original litter average.*
2) *A new average is calculated.*
3) *Same-sex siblings with a weight closest to this new average are defined as NBW controls.*

Allopurinol: a preventive therapy?

IUGR and the brain

A foetus that suffers from an inadequate nutrient or oxygen supply due to placental insufficiency will react by slowing down its growth rate. This can increase its chance of survival and prepares for extra-uterine circumstances (Barker. 1997, Gagnon. 2003). Also, some foetuses have, to a certain extent, the ability to protect their brain against reduced growth by a phenomenon called 'foetal brain sparing' (Barker. 2004, Cheema et al. 2009, Klaric et al. 2012). This adaptive reaction of diverting more blood to the brain, at the expense of blood trunk supply, does not completely spare the brain and there might still be consequences for later behaviour (Roza et al. 2008). Sometimes, based on head-body size ratio, IUGR children without brain sparing are referred to as symmetrically growth restricted, whereas children with brain sparing show asymmetrical growth (e.g. Klaric et al. (2012)). However, most studies do not distinguish between asymmetrical and symmetrical growth restriction.

Brain hypoxia

In most cases it is likely that the cognitive deficits from which children (and probably piglets) with LBW may suffer originate from mild or more severe brain damaging hypoxia during pregnancy. Although this is the prevalent theory, it is not well understood and the exact underlying mechanisms are unclear (Mallard et al. 2000, Yanney and Marlow. 2004) Deprivation of oxygen may lead to neuronal cell damage or even cell death (de Haan and Hasaart. 1995, Peeters and van Bel. 2001). Foetal distress may ensue when the foetus is not able to cope with the insufficient oxygen supply and the compensatory mechanisms are inadequate (Sankaran and Kyle. 2009). Unlike acute birth asphyxia/hypoxia, it is hypothesized that IUGR foetuses undergo longer but milder periods of oxygen deprivation. Multiple periods of oxygen deprivation and of re-oxygenation may occur. During re-oxygenation additional damage to brain cells is caused by the free radicals produced (Biri et al. 2007, Peeters and van Bel. 2001, van Bel et al. 2006).

During cerebral hypoxic periods and subsequent re-oxygenation a cascade of conversions takes place. Adenosine-5'-triphosphate (ATP) is degraded into

hypoxanthine via several steps. Hypoxanthine accumulates during the actual hypoxic period and is then further converted into xanthine and uric acid. The enzyme xanthine oxidase (XO) and oxygen are needed in this step. (Kaandorp et al. 2010a, Pacher et al. 2006) The conversion process leads to the formation of large amounts of superoxide free radicals / reactive oxygen species (ROS). Additionally, acids freed from cell membranes that were damaged during the hypoxic period can lead to the formation of ROS. This chain of reactions in turn may lead to further formation of cell damaging free radicals (Kaandorp et al. 2010a).

Preventive therapies

As mentioned previously, due to close prenatal monitoring, foetuses suffering from IUGR are detected during pregnancy in most cases in developed countries (Chaddha et al. 2004). Multiple foetal heart rate and ultrasonographic (with additional Doppler) examinations aid diagnosis during the 2nd or 3rd trimester of pregnancy. It is tempting to start a treatment to prevent damage, as soon as IUGR has been diagnosed. Unfortunately, pharmacological therapeutics that cure or prevent IUGR are not yet available.

In foetuses acutely asphyxiated during the parturition process, treatment is often delayed till after birth or is commenced during delivery when asphyxia is suspected. As the hypoxic process has already started, this treatment is not always optimal (Kaandorp et al. 2010b). When an IUGR foetus is diagnosed during pregnancy, prenatal pharmacological treatment could begin immediately and continue until delivery or until the neonate is a few days old. It has been hypothesized that, if the formation of damaging effects of hypoxia-related free radicals could be prevented or reduced, neurological dysfunctions in these children could also be prevented or at least ameliorated.

Allopurinol

The compound allopurinol (1,5-dihydro- 4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one) was found to inhibit XO (see 'Brain hypoxia') (Elion. 1988). It is expected that allopurinol has neuroprotective effects in foetuses at risk of acute or chronic hypoxia. Allopurinol (an isostere of hypoxanthine) is rapidly oxidized by XO *in vivo* to its metabolite oxypurinol (an isostere of xanthine), which also inhibits XO. At lower concentrations allopurinol is a substrate for and a competitive inhibitor of the XO enzyme. At higher concentrations it is a non-competitive inhibitor (Pacher et al. 2006) and scavenges free radicals. The metabolite oxypurinol is also a non-competitive inhibitor of XO (Pacher et al. 2006).

Based on studies assessing the neuroprotective capacities of allopurinol in foetuses and neonates suffering from acute asphyxia during the parturition process, it was suggested that treatment could have the most beneficial effect if it would be administered as early as possible and only when the level of asphyxia is not too severe (i.e. that there is not yet irreversible damage) (Kaandorp et al. 2010b). As it is expected that IUGR is caused by a chronic, but in general mild intra-uterine hypoxia, allopurinol could be a suitable candidate for preventive treatment of foetuses diagnosed for IUGR.

To be applied as a prenatal therapy it is necessary that the substance can be administered via the mother with a repeated dosing regimen. Therefore, the practicality and invasiveness of intake of the compound is of great importance, and oral administration is preferred. Allopurinol readily crosses the human and pig placenta and does not interfere with the parturition process if administered acutely during parturition or several days earlier (Boda et al. 1999). These characteristics suggest that maternal chronic prenatal allopurinol administration may be an interesting therapeutic strategy for treating diagnosed IUGR.

Studies looking at the neuroprotective capacities of allopurinol and its active metabolite oxypurinol in, for example, pig, sheep and human foetuses have found reduced free-radical formation (Boda et al. 1999, Masaoka et al. 2005, Peeters-Scholte et al. 2003), inhibition of xanthine oxidase (Dallwig. 2010, van Bel et al. 1998) and scavenging of toxic free radicals (Moorhouse et al. 1987). There is a lack of information about the therapeutic range for neuroprotection via chronic oral allopurinol administration. Torrance and colleagues (2009) suggest minimal plasma levels for neuroprotection to be >2 $\mu g.ml^{-1}$ for allopurinol and >4 $\mu g.ml^{-1}$ for oxypurinol.

Aim and structure of the thesis

The final aim of this thesis is to study the cognitive performance of piglets suffering from intra-uterine growth restriction and to investigate whether chronic allopurinol treatment is safe and efficient in preventing possible adverse consequences of IUGR on cognition, using piglets as subjects.

To investigate the cognitive performance and possible treatment of piglets suffering from IUGR, more specific questions need to be addressed first:

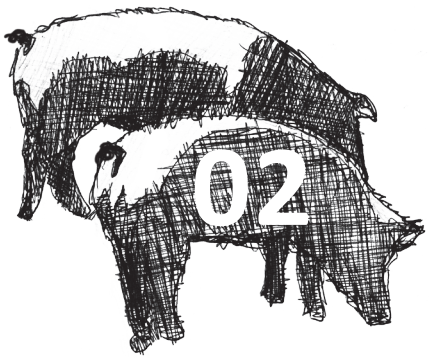
1. What cognitive tests for pigs are available and what is necessary to establish a valid, robust and reliable cognitive test for this species?
2. Does low-birth-weight in piglets — as a read-out parameter for intra uterine growth restriction — influence their later cognitive performance and are we able to measure this?
3. Could allopurinol be applied as a safe and efficient prenatal therapy for preventing brain damage in LBW piglets?

This thesis is split up in three parts, each addressing one of these questions. **Part 1** explores what is known about the cognitive abilities of pigs in general and how to study pig cognition in the field of animal welfare and biomedical research (chapter 2).

This leads to **part 2**; the development of two different learning and memory tasks for pigs, namely a visual discrimination task (chapter 3) and a cognitive spatial pig holeboard task (chapter 4). Low and normal-birth-weight piglets are tested in both tasks. We conclude that the cognitive pig holeboard is the best suited of the two tasks, and therefore validation and automation steps are taken in order to improve the reliability of the test equipment and to test different pig lines (chapter 5 and appendix I).

Part 3 of this thesis describes a final practical application of both the low-birth-weight piglet model and the cognitive pig holeboard. The substance allopurinol is administered prenatally to low and normal-birth-weight piglets via the sow as a putative therapeutic strategy for preventing brain damage (chapter 6 and 7) in the growth restricted animals. Correct dosage of allopurinol is first explored in a small-scale pharmacokinetic experiment with allopurinol in pregnant sows and piglets (integrated in chapter 6).

The combined results of the separate experiments outlined in chapter 3-7 are discussed in the general discussion at the end of this thesis (chapter 8).



Assessing learning and memory in pigs

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Abstract

In recent years, there has been a surge of interest in (mini) pigs (Sus scrofa) as species for cognitive research. A major reason for this is their physiological and anatomical similarity with humans. For example, pigs possess a well developed, large brain. Assessment of the learning and memory functions of pigs is not only relevant to human research but also to animal welfare, given the nature of current farming practices and the demands they make on animal health and behavior. In this article, we review studies of pig cognition, focusing on the underlying processes and mechanisms, with a view to identifying. Our goal is to aid the selection of appropriate cognitive tasks for research into pig cognition. To this end, we formulated several basic criteria for pig cognition tests and then applied these criteria and knowledge about pig-specific sensorimotor abilities and behavior to evaluate the merits, drawbacks, and limitations of the different types of tests used to date. While behavioral studies using (mini) pigs have shown that this species can perform learning and memory tasks, and much has been learned about pig cognition, results have not been replicated or proven replicable because of the lack of validated, translational behavioral paradigms that are specially suited to tap specific aspects of pig cognition. We identified several promising types of tasks for use in studies of pig cognition, such as versatile spatial free-choice type tasks that allow the simultaneous measurement of several behavioral domains. The use of appropriate tasks will facilitate the collection of reliable and valid data on pig cognition.

Keywords: Pig – Cognition – Learning – Memory – Welfare – Model animal

Introduction

Over the past 100 years, scientists have shown that a number of animal species have substantial cognitive (i.e., learning and memory) abilities (Broom and Zanella. 2004, van der Staay. 2006). In recent decades, scientists have primarily focused on rodents and, to a lesser degree, on primates, species considered relevant to human research (van der Staay. 2006). When assessing the utility of animal models for investigating cognitive functions such as learning and memory, it is necessary to evaluate which species and what tests are most suitable, produce valid results, and allow generalization to humans (referred to as translational research; Markou et al. (2009)). In general, learning and memory are considered to require higher brain functions and are not merely the acquisition of a series of elicited responses (Kirsch et al. 2004). This is why it is so important to select the correct parameters so that learning is measured rather than more effortless or less deliberate types of performance (for which other strategies are adopted) (Kratzer. 1971). Pigs are cooperative animals and learn classical and operant conditioning tasks rapidly (Baldwin. 1969, Baldwin and Stephens. 1973, Chaput et al. 1973, Kratzer. 1971). They are generally seen as being 'intelligent' by the public, probably because they can be trained. For example, Breland and Breland (1915) successfully trained pigs for some pig shows, based on B.F. Skinners' operant conditioning methods. Yet despite the growing literature on the cognitive abilities of pigs (including miniature or minipigs) (Ferguson et al. 2009), experience with and knowledge of this species as an animal model are still limited (Hagl et al. 2005). Pigs and minipigs have several advantageous characteristics, such as greater similarity to humans than rodents have, which might favor their use instead of—or alongside—other model species. For this reason, experimental data produced over the past 40 years should be verified and steps taken to advance research in the field of pig cognition. Animal welfare scientists, while sharing the opinion that pigs have considerable cognitive abilities, look at cognitive research in pigs from a different point of view from that of biomedical researchers. Their aim of studying this species is to become more aware of pigs' cognitive abilities and sensory capacities, ultimately to improve the welfare of this intensively kept farm animal (Meehan and Mench. 2007, Toates. 2004). An additional aspect is that the public's perception of the intelligence of an animal influences the importance attached to its welfare, and many people (consumers) consider farming practices that result in poor animal welfare to be unacceptable (Broom. 2010). Cognitive ability should also be considered when designing methods of enriching the environments of captive animals. The aim of this paper is to review the literature on studies of learning and memory in pigs from these two perspectives, focusing on the different types of tasks used (operant, spatial and recognition tasks, and tasks that assess observational learning and awareness) and distinguishing between appetitively and aversively motivated tasks. We also review the conditions under which tests should be performed in the future.

The pig as model species in biobehavioral research

Although the scientific advantages of using the pig, in particular the minipig, as animal model in biomedical research have long been recognized (e.g. Baldwin and Stephens (1973) and Chaput et al. (1973)), there has been a recent revival

of interest in the pig as model of human disease. The pig has the potential to fill the gap between preclinical studies with rodents and clinical trials in humans (de Groot et al. 2005, Lind et al. 2007, Nunoya et al. 2007, Vodicka et al. 2005). Its organ size, body mass, and physiology strongly resemble those of humans (Sachs. 1994, Schook et al. 2005), and the immune system of pigs is more similar to that of humans than it is to the immune system of rodents (80% similarity of the compared variables between pigs and humans versus 10% between mice and humans) (Schook et al. 2005). Unlike rodents, which have a lisencephalic brain, pigs have a gyrencephalic brain, as do humans.

The brain of an average adult pig weighs up to 180 g and that of minipigs 70–80 g (Hofman. 1985). The relatively large brain of pigs makes it suitable for imaging studies, particularly in young animals (Arnfred et al. 2004, Danielsen et al. 2001). For example, positron emission tomography (PET) studies have investigated aromatic amino acid decarboxylase activity in the brain of newborn piglets (Bauer et al. 2002), magnetic resonance imaging (MRI) has been used to study the temporal expansion following cerebral contusion (Zhang et al. 2008), and functional magnetic resonance imaging (fMRI) has been used to study activity in the cortex, cerebellum, and brainstem following visual stimulation in postnatal piglets (Fang et al. 2006). In addition, the sequence and chromosome structure homology of the pig genome show strong similarity with those of the human genome (Chen et al. 2007, Lunney. 2007, Petersen et al. 2009). However, the full potential of pig-based models for investigating the function/dysfunction of the nervous system in the regulation of normal and abnormal behavior has not yet been fully explored (Nielsen et al. 2009, Schook et al. 2005). Overall, it could be said that the pig shows several favorable physical characteristics, including suitability for advanced imaging techniques that support its use in research into human disease (Lind et al. 2007).

Traditionally, behavioral studies have used rodents because of the availability of numerous well-validated tests and models (Kornum et al. 2007), and the ease and cheaper costs of housing rodents compared with larger species. However, the pig may be a good non-primate, non-rodent species for biomedical research, for studying the effects of a wide range of clinical and behavioral stresses (Chaput et al. 1973), for screening in vivo receptor profiles of drugs (Lind et al. 2004), and for verifying neurological syndromes (e.g. MPTP-induced Parkinsonism syndrome; Mikkelsen et al. (1999)). Pig models have been used to address lifestyle factors (e.g., stress, drugs, and abuse; Schook et al. (2005)), diabetes (Larsen and Rolin. 2004), and human brain disorders (Nielsen et al. 2009, Moustgaard et al. 2005).

Another important consideration is that pigs are relatively inexpensive compared with primates (Mikkelsen et al. 1999). Several domestic pig lines and a smaller number of minipig breeds are now commercially available from specialized breeders. However, the housing, physiology, and behavior of domestic pigs and minipigs have not yet been directly compared. The nature of the domestic pig and minipig breeding programs is different, especially with regard to weight gain. The Göttingen Minipig is bred to be a small and light laboratory animal (mature animals weigh around 30–35 kg, if they have been put on a calorie-

controlled diet) and shows nearly linear growth in the first 160 days of life. In contrast, fattening pigs (weighing 117–138 kg when 26 weeks old, depending on their feeding regime) gain little weight during the first 7 weeks, but thereafter gain weight rapidly (Köhn et al. 2007). Researchers should be aware of these and other differences between the two types of pig when comparing data. Indeed, further research on potential differences is urgently needed.

Learning and memory in relation to welfare

Greater insight into pig cognition is needed not only with regard to biomedical research, but also with regard to improving pig welfare. Both research lines will benefit from the development of reliable and validated tests for studying pigs' cognitive abilities. Indeed, Duncan and Petherick. (1991) stated ''animal welfare is dependent solely on the mental, psychological and cognitive needs of the animals concerned''. Farm animals, including pigs, are inadvertently exposed to many learning and memory challenges (Held et al. 2002), and knowledge of species specific learning abilities and environmental preferences is expected to contribute to improvement of housing conditions, management, and handling routines, and hence animal welfare (Baldwin and Meese. 1977, Boissy et al. 2007, Tanida and Nagano. 1998, van Rooijen. 1982, Wechsler and Lea. 2007). Situations that adversely affect farm animal cognition can trigger stress responses (possibly associated with suffering) and can negatively affect productivity (Held et al. 2002). The capacity of an animal to cope with its housing conditions may be influenced by its learning abilities (Wechsler and Lea. 2007).

A range of cognitive factors appear to influence emotions or emotional processes in animals (a topic nowadays of great importance when assessing animal welfare) (Ohl et al. 2008). More complex emotions are the result of interactions between cognitive and emotional processes that are needed for the evaluation of perceptual information (Ohl et al. 2008, Paul et al. 2005). Vice versa, the emotional state of an animal can also influence its cognitive functioning, ''judgment of stimuli'' (Mendl et al. 2009). Consequently, questions about farm animal welfare cannot be tackled without a thorough understanding of the fundamental psychology and behavior of these animals (Curtis and Stricklin. 1991), an aspect that has long been neglected in farm animals (Puppe et al. 2007), but which influences our attitude to these animals. Nowadays, there is a growing public interest in and discussion of animal welfare issues, and cognitive research in farm animals may provide information relevant to these discussions (Mendl and Paul. 2004).

Our aim is to review the biomedical and animal welfare literature with a view to facilitating the selection or design of appropriate tasks for pig cognition research. We hope it will prompt collaboration between scientists in both fields, to improve biomedical research models and animal welfare.

Implementation of cognitive tasks

Research into the effects of experimental interventions on cognitive processes in pigs necessitates the development of reliable and valid learning and memory tests (e.g., van der Staay (2006)). One of the reasons why pig studies are under-represented in biomedical behavioral research is the lack of well-standardized

and validated tasks. A main task of behavioral scientists is to develop or adapt existing tests to generate valid and sensitive test paradigms applicable to most commonly used model animals in addition to pigs and/or suitable for behavioral characterization (phenotyping).

Behavioral tasks for pigs should in general fulfill a number of criteria:

1. healthy, unimpaired animals should be able to acquire/perform the task;
2. the task should allow a detailed analysis of pigs' behavior, i.e., it should preferentially provide indices for different behavioral domains (cognitive, sensory, motor, or motivational components) (Wainwright and Colombo. 2006);
3. the task should be as stress-free as possible (for both the experimental animal and the experimenter; except if measuring the effects of stress is an explicit aim of the experimental procedure);
4. the task should preferentially tap ecologically relevant behaviors (e.g., to prevent mismatches between the task and the adaptive mechanisms and available behavioral repertoire of the species) (Koolhaas et al. 2006);
5. the task should be standardized in order to enable comparisons between studies within and across laboratories (van der Staay et al. 2010);
6. the task should, wherever possible, be automated in order to eliminate variability between observers, and to allow fine-tuned analyses;
7. the task should allow investigation of developmental effects (early ontogeny, aging) and should preferentially be suited for repeated testing (van der Staay. 2002) in order to allow longitudinal studies;
8. the task should be complex and sensitive enough to capture subtle differences in cognitive abilities (Friess et al. 2007, Hagl et al. 2005, Laughlin et al. 1999).

The above criteria are general criteria for test paradigms. Clearly, some criteria will be more relevant than others, depending on the specific research questions (biomedical or welfare related) and hypotheses tested. A multi-layered approach is needed to cover the full range of pig behavior, because cognition is not a unitary function but involves multiple and dissociable systems that interact in cognitive processes. This should be kept in mind when designing and interpreting studies of cognitive functioning (Wainwright and Colombo. 2006).

Species-specific opportunities and constraints

Pigs are clearly different from rodents. It is important to take the species-specific abilities and constraints into account when developing new tasks or applying existing ones for pigs. In this context, we will briefly discuss the general characteristics of the domestic pig from an evolutionary point of view and give an overview of pigs' known sensory capacities.

General features of the domestic pig

The behavioral traits of domestic pigs, which were domesticated around 9,000 years ago (Hemmer. 1990), closely resemble those of its ancestor, the wild boar (Sus scrofa). Thus, study of wild and/or feral pigs provides insights into the behavior of the domestic pig (Graves. 1984). Ethological studies have shown that wild boars, which are highly social and omnivorous (Graves. 1984, Gustafsson

et al. 1999), concentrate their daily activity into several main periods, generally synchronized with sunrise and sunset (Mauget. 1984), depending on season, predator pressure, and food availability. Wild boars are active for about 65% of the time (Graves. 1984), and although mainly diurnal, they can easily shift to nocturnal activity (Jensen. 2002). During foraging, they move between different feeding areas while grazing, browsing, or, more commonly, rooting with their snout. Their muzzle (a flattened, tough, rounded disk) searches for food on and under the surface, and pigs generally move with their nostrils close to the ground (Graves. 1984). Because pigs lack sweat glands, wallowing in mud or water is a common behavior to decrease body temperature (Jensen. 2002).

The natural behavior of feral pigs and their ancestors gives us some insights into behaviors and aspects of domestic pigs that are of significant importance (e.g., rooting, social companionship, and the lack of sweat glands) to their well-being and which should be taken into account when housing experimental animals and designing or selecting suitable tasks. Typical behavior such as rooting could be used as stimulant to motivate animals to perform a task, in addition to food reward. Because most tasks are performed by individual animals, it is essential to habituate these social animals to being alone in the test environment. Fear, stress, or arousal can influence performance and decrease motivation (this will be discussed later on). Keeping the pen mates of the tested individual close to the experimental set-up could help to decrease arousal, because pen mates are within hearing and smelling range of the test pig. In cognitive tests in which the pig's emotional state plays a role, communication and/or pheromone signaling between the test pig and the waiting pen mates may influence test performance. Moreover, it is conceivable that the testing order affects physiological and possibly behavioral measures (e.g. within-cage order effects of testing are found in mice (Arndt et al. 2009)).

Sensory capacities

Visual and olfactory capacities

Knowledge about the visual and olfactory capacities of the pig is limited, and the available results are contradictory. Pigs can learn olfactory discrimination tasks faster than visual discrimination tasks (Croney et al. 2003, Lind et al. 2007). However, despite large variations between individual pigs, (Tanida and Nagano. 1998) found visual as well as auditory cues to be more important than olfactory cues when pigs had to discriminate between people. Pigs detect odor cues very well, as evidenced by their use in truffle hunting (Pacioni. 1986). Therefore, it is important to control odor cues so that the test can discriminate between physiological innate responses and learned behavior (Hagl et al. 2005).

Less is known about the visual capacities of the pig, and only a few studies have addressed pigs' ability to distinguish details and shapes (visual acuity). Zonderland et al. found pigs to perform poorly when distinguishing smaller symbol sizes at close (\600 mm) range, but also found a large individual variation. Their animals failed to discriminate between visual cues smaller than 20 mm and the minimum-distinguishable visual acuity was about 0.001–0.03 (measured

corresponding to (Entsu et al. 1992), lower than that of cattle and humans. In contrast to Graf (1976), Zonderland et al. (2008) did not find a strong decrease in visual acuity below 12 lx (lux) for black-and-white cues. As regards pig color vision, Tanida and Nagano (1998) found that two sows could discriminate blue from red and green, but were unable to discriminate red from green (all with the same luminosity), whereas two other animals failed to discriminate red or green from gray but could distinguish between blue and gray. The results show that pigs discriminate blue from other colors on the basis of hue rather than brightness and suggest that pigs are red–green color blind or can poorly discriminate between these colors.

Auditory capacities

The pig's hearing range is from 42 through 40,500 Hz (Heffner and Heffner. 1992), and exceeds the human hearing range (31–17,600 Hz). This increases the risk of pigs being unwittingly exposed to aversive environmental noise (experimental machinery, etc.). Social vocalization plays a role in communication and recognition between pigs and can provide complex information about the identity of the sender and its arousal state (Held et al. 2009). This knowledge should be kept in mind when testing pigs in an experimental room with other animals present within hearing distance.

Gustatory preferences

Pigs appear to like sweet tastes (Kennedy and Baldwin. 1972). Glaser et al. derived more specific gustatory information by testing 75 pigs in an adapted Richer-type drinking test. None of the pigs drank a bitter-tasting quinine hydrochloride solution (49 mg/l), but preferred different carbohydrate solutions to water, with sucrose being the most preferred. Seven polyols were preferred to water, with xylitol being the most preferred. Of twelve artificial or natural compounds considered sweet by humans, only acesulfame-K, alitame, dulcin, saccharin, and sucralose-D were able to elicit a reference response in pigs (Glaser et al. 2000). In a further study, 120 pigs were tested with 60 compounds perceived as sweet by humans. Lugduname and carrelame (both guanidinoacetic acid derivatives) are considered the sweetest by humans and proved to be the two most preferred compounds in pigs. (Nofre et al. 2002)

Summary

Although pigs are probably red–green color blind and probably cannot distinguish very small symbol sizes, too little is known about their visual capacities, and this has implications for task design (e.g., is poor performance caused by poor visual capacities or cognitive limitations?). Images can be projected onto touch screens during discrimination experiments or similar tasks with humans, primates, or chicken as subjects, but before we can use this approach with pigs (or use images in general), we need to know more about their visual capacities, to exclude possible false-negative results because pigs are physically incapable of performing the task. As pigs' auditory acuity is better than that of humans, tones can serve as discriminative stimuli, or as secondary or conditioned reinforcer. However, it is important that researchers are aware that pigs hear, and may be disturbed by, sounds that are inaudible to humans. Beside auditory reinforcers, sweet solutions

can be used as effective reinforcers, and quinine can be added to food if an aversive taste experience is needed. Somatosensory information concerning pigs is as yet lacking, and should be further investigated.

Reinforcements

Food rewards, such as pieces of apple, chocolate raisins, M&M chocolates, sow rolls, commercial pellets, dog biscuits, or milk replacer (for piglets) are most commonly used as reinforcers in appetitively motivated research (Croney et al. 2003, Hagl et al. 2005, Held et al. 2001, Laughlin et al. 1999, Moustgaard et al. 2005, Siegford et al. 2008, Tanida and Nagano. 1998). In order to increase motivation and the reinforcing value of food rewards, food deprivation is often applied when testing pigs (Held et al. 2001, Held et al. 2005, Laughlin et al. 1999, Laughlin and Mendl. 2000, Laughlin and Mendl. 2004, Mendl et al. 1997, Moustgaard et al. 2002, Nielsen et al. 2009, Spinka et al. 1998), but is not always necessary (Arts et al. 2009, Ferguson et al. 2009). Lack of appetite (e.g., caused by treatment with drugs that can induce nausea or anorexia as side effect) (Chaput et al. 1973) should be borne in mind, as this may make the use of food rewards impossible.

Non-food reinforcers have also been used with success. Pigs are social animals that are motivated to perform a task in which access to the group (or to the sow in the case of preweaning piglets) serves as reinforcer (Siegford et al. 2008, van Rooijen. 1982). Another type of reinforcer that has proved effective is light in darkness (Baldwin and Meese. 1977, Chaput et al. 1973). The latter seems to be a mediocre reinforcer, but becomes more important when olfaction is removed by bulbectomy (Baldwin and Meese. 1977). A dry area —in case of a water maze— (Siegford et al. 2008) and heat (Baldwin. 1979, Baldwin and Meese. 1977) are also effective reinforcers.

It is also important to consider the way in which the reinforcer is applied and how access to it is achieved. For example, Baldwin and Meese (1977) found pigs to work more consistently if they could push a beam with their snout intermittently, rather than constantly, to obtain light. As described in Sect. 2.1, pigs have evolved to use their nose to seek and root; their legs and hooves are not designed to make subtle motor movements and their body is not that athletic (e.g., compared to most primates' bodies). Thus, in order to keep animals motivated and to facilitate performance without duress, pigs should be allowed to use their snouts to reach the reinforcer or to manipulate a lever or similar mechanism. Tasks should be designed such that the required action matches one or more of the pig's natural behaviors and that the pig naturally 'understands' the task, i.e. does not require extensive trial and error trials. This facilitates task learning and will keep the animal more motivated.

Tests for assessing learning and memory in pigs

Underlying learning mechanisms

Learning and memory tasks, based on different underlying learning methods, have been administered to pigs and are briefly defined here. Examples of research involving pigs and different learning methods will be presented in subsequent sections. In Table 1, these examples are outlined point-by-point, summarizing

relevant information about subjects and materials (e.g., number and age of animals, and reinforcer used).

Classical or Pavlovian conditioning studies in pigs, which imply learning about relations between stimuli, with one stimulus signaling the occurrence of the other (Rescorla. 1988), were used in the early 1900s. Although Pavlov apparently thought that pigs could not be used as experimental subjects (Moore and Marcuse. 1945), evidence has since accumulated that pigs can easily be conditioned (Kratzer. 1971), using classical conditioning methods. Yet only a few classical conditioning studies have used pigs, as reviewed in the 'Operant conditioning tasks'.

When the response to a stimulus is followed by a reinforcer, the probability that the response will be made is increased. This is called operant conditioning or instrumental learning (Rescorla. 1988). Yerkes and Coburn (1915) were probably the first to study operant conditioning in pigs, and since then operant conditioning has become a commonly used conditioning technique. Different types of reinforcers, both aversive and appetitive, have been used.

Spatial tasks

Spatial learning and the memory ability of animals can be assessed using different types of mazes, the so-called sequential choice or 'alley' mazes and 'free-choice' mazes. The alley mazes consist of a fixed starting position and one correct route to a fixed goal position, where incorrect alternatives such as visits to blind alleys or going back must be avoided. In contrast to 'alley' mazes, in 'freechoice' spatial discrimination tasks (Bouger and van der Staay. 2005, Crannell. 1942, Lachman and Brown. 1957), rewards can be found in different places, and the animal is free to visit and revisit these baited places and unbaited alternatives, in whatever order it wishes. Once an animal has visited a place and consumed the food pellet, its revisits to the same location remain unreinforced. The most efficient behavior is to visit only baited locations, and to visit them only once. In spatial memory tasks, an animal must remember a list of places already visited in order to avoid revisits. This list of visits is held in the working memory (Olton and Samuelson. 1976), and the information it contains is relevant only within a specific trial. The reference memory (Olton and Samuelson. 1976) holds trial-independent information about, for example, the locations where the food reward can be found. Working memory and reference memory can be assessed simultaneously in freechoice mazes. As most variants of T- and Y-maze tasks for pigs are not based on the orientation of the animal in relation to the space it finds itself in, the pig variants of these tests will not be considered as spatial but as operant (or in some cases social) tasks.

Recognition tasks

The object recognition test (ORT) was developed by Ennaceur et al. for assessing 'trial-unique' memory in rodents (Ennaceur et al. 1989, Ennaceur and Delacour. 1988, Ennaceur and Meliani. 1992), based on the known preference of rodents to explore unknown objects more than familiar ones. The ORT provides measures of exploration, habituation, and discrimination, i.e., non-cognitive effects of experimental manipulations can be distinguished from effects on memory performance (Sik et al. 2003).

Similar results are obtained when the ORT is used to test social recognition and memory. Thor and Holloway (1982) studied this behavior in rats by re-exposing animals successively to familiar or unfamiliar conspecifics with differing inter-exposure intervals, and the cumulative duration of investigatory behavior was measured during re-exposure. In pigs, social recognition tends to be studied in a simultaneous setting (i.e., exposing the animal to different conspecifics at the same time), in a Y-maze (Kristensen et al. 2001, McLeman et al. 2005). For example, the recognition of humans by pigs was investigated by displaying familiar and non-familiar humans simultaneously in a Y-maze (Koba and Tanida. 1999, Tanida and Nagano. 1998). Another variant of social learning studied in pigs is observational learning (e.g., Held et al. (2000) and Held et al. (2001)). Held describes it as studying social tactics (i.e., the 'exploitation of knowledge of others' or 'deceptive tactics'). Although it is not behavioral imitation as such, this definition comes close to the definition 'the capability to imitate a demonstrator's behavior', a type of learning often studied in monkeys and apes (Choleris and Kavaliers. 1999).

Awareness

Animals' awareness or the ability to perceive, feel, or be conscious of events or objects can be studied by investigating their use of tools. Such studies usually involve primates and corvids, which are physically equipped to use tools with their paws or beak, whereas pigs are not capable of doing this. However, mirrors, and the information obtained from them, were recently used to demonstrate awareness in pigs (Broom et al. 2009).

Classical conditioning tasks

Appetitively motivated tasks

In a small-scale study with two pigs, Moore and Marcuse (1945) examined 4 types of conditioning, including classical conditioning. They attempted to establish a 'conditioned salivary response' in an experimental setting similar to that used by Pavlov. Pigs equipped with a parotid fistula were trained to tolerate restraint on a platform. A tone (the conditioned stimulus, CS) was presented, immediately followed by food (the unconditioned stimulus, US). Both pigs established a stable, but not equally large, conditioned salivary response. In addition, the response was more profound in a laboratory setting than during feeding in the home pen, which is a less-controlled environment. Feeding time was signaled about 5 min in advance and elicited a conditioned salivary response in 100 and 75% of the trials in the laboratory, but only in 67 and 6.7% of the trials performed in the home pen.

Aversively motivated tasks

Noble and Adams (1963) examined the effect of interval length between a CS and an US on classical conditioning performance using Duroc pigs in two different experiments. In the first experiment, the CS–US interval ranged between 0.5 and 2 s, the CS was an increase in illumination, and the US was an electric shock to a hind leg. In the second experiment, the CS was a combination of an increase in illumination and a vibratory–auditory cue from a buzzer strapped to the neck behind the subjects' ear, the CS–US interval was 1, 2, 4, and 8 s), and the US was as in the first experiment. The conditioned response

(CR) of the animals after several trials was described as a 'bracing' posture. The CR was found to be more pronounced with increasing CS–US interval. This might partly be caused by the increased opportunity to respond to the prospective US.

These experiments support the notion that pigs can be classically conditioned using an aversive or appetitive US. However, it has not been proven that the length of the CS– US interval is the sole determinant of the presence and intensity of the CR. Probably because classical conditioning experiments are of limited interest to cognitive researchers, these relatively sensitive and automated experiments have not been followed up.

Operant conditioning tasks

Most operant conditioning studies have been performed using either positive (appetitive) or negative (aversive) reinforcers. Yerkes and Coburn (1915) decided to make use of both variants in an operant conditioning experiment with two Chester White pigs. The apparatus, situated in a meadow, consisted of 9 similar boxes and food troughs. The experimenters presented 4 different 'problems' to the pigs. The correct operant response required to receive a food reward was to choose the correct entry door out of several opened boxes. A wrong entry was punished with 1 min of confinement in the box. Entry into the correct box led to a filled food trough. The pigs acquired the task, but when one 'problem' was replaced by another problem, the second problem was solved more slowly, probably caused by proactive interference. Yerkes and Coburn commented that this research was a clever way to gain information about the ability of pigs to adjust themselves to fairly simple, but novel, situations (this is what they named ideational problem solving). It was several decades after this seminal study that learning experiments with pigs were repeated using an operant conditioning setting.

Appetitive learning

In the 1960s and 1970s, Baldwin et al. studied the pig extensively in several operant conditioning experiments. In 1973, they trained, within an hour, pigs that were loosely restrained in a metal stand to press levers with their snout for a food reward. The authors suggested that the pigs acquired the task so rapidly because the experimental environment was not new to them: the pigs had previously been trained in a thermal reinforcement experiment. (Baldwin and Stephens. 1973)

Food-rewarded panel switching was used by Kennedy and Baldwin (1972) to study taste preferences in pigs. Differing amounts of nutritive and non-nutritive sweeteners were added to water, and a progressive ratio (PR) schedule of

Table 1. Cognitive tasks performed in pigs. Task: Name of the experiment performed. **Tested cognitive ability/ abilities:** Type of cognition measured during the experiment. **N:** Amount of animals applied during the experiment. **Sex:** F = female/ sow, M = male/ intact boar, B = castrated male/ barrow. **Reinforcer:** Type of reinforcement applied. **Age:** Age of animals at the beginning of the experiment, or if unknown, weight of animal at the beginning of the experiment. **Food restriction schedule:** If applied, type of restriction schedule. **Author:** Researcher(s) performing the experiment.

Task	Tested cognitive ability/ abilities	N	sex	reinforcer	age (or weight)	food restriction schedule	Author
Classical conditioning tasks							
- Appetitive learning							
Conditioned salivary response	Association learning	2	F	food	6 weeks	unknown	Moore and Marcuse, 1945
- Aversive learning							
Conditioned aversive response	Association learning	50-64	?	-	75-135 days	-	Noble and Adams, 1963
Operant conditioning tasks							
Multiple choice apparatus	Ideational problem solving	2	B/F	food	2 months	unknown	Yerkes and Coburn, 1915
- Appetitive learning							
Conditioned suppression of operant responding	Learning ability	12	B/F	food	2-4 months	unknown	Baldwin and Stephens, 1973
Lever pressing	Hierarchy behaviour and social learning	64	B/F	commercial pellets	20-40 kg	abstention 24h pre-testing	Baldwin and Meese, 1979
Panel-switching	Preference testing	66	B/F	sweetened water	2-4 months	fed 1x daily	Kennedy and Baldwin, 1972
Lever pressing	Learning ability	84	M/F	unknown	15-17 weeks	at libitum	Sneddon et al., 2000
Lever pressing	Measure of motivation	6	F	unknown	4.5 months	fed 2x daily	Ferguson et al., 2008
Temporal Response Differentiation Training	Time perception	3	F	unknown	4.5 months	fed 2x daily	Ferguson et al., 2008
Incremental Repeated Acquisition	Learning ability	3	F	unknown	4.5 months	fed 2x daily	Ferguson et al., 2008
Discrimination reversal test	Reversal learning	34	M/F	food	> 35 days	unkn. deprivation schedule	Lien and Klopfer, 1978
Reversal learning	Learning ability	60	B/F	commercial pellets	8 weeks	at libitum	Bolhuis et al., 2004
T-maze	Discrimination learning	4	F	food	21-42 days	unknown	Tanida et al., 1991
Y-maze	Discrimination/ recognition learning	5	M	raisins	8 weeks	unknown	Tanida and Nagano, 1998
Y-maze	Discrimination/ recognition learning	6	F	raisins	8 weeks	fed 2x daily	Koba and Tanida, 1999
Standard human approach test	Association learning	24-36	F	commercial finisher ration	17-23 weeks	fed 1x daily	Hemsworth et al., 1996
Discrimination learning	Spatial, visual and olfactory learning	4	B	milk-bone dog biscuits	2.5-3 years	no restrictions	Croney et al., 2003
Eight-arm radial maze	Discrimination learning and memory	53	unkn.	milk replacer	3 days	fed 4x daily	Wang et al., 2007

Task	Tested cognitive ability/ abilities	N	sex	reinforcer	age (or weight)	food restriction schedule	Author
Set-shifting procedure	Spatial, visual, reversal and extra-dimensional learning	16	M/ F(c)	M&M chocolates	4 months	70% of daily ration	Moustgaard et al., 2004
Conditional go/ no-go task	Learning ability	14	M/ F(c)	M&M chocolates	5-5.5 months	70% of daily ration	Moustgaard et al., 2005
Food covering	Discrimination learning (non-visual)	20-25	F	milk replacer	1-12 days	unknown	Friess et al., 2007
Glass barrier task	Problem-solving skills	20-25	F	milk replacer	1-12 days	unknown	Friess et al., 2007
- Aversion learning							
Avoidance conditioning	Learning ability	84	B/F	unknown	40/ 80/ 150 days	unknown	Kratzer, 1969
Avoidance conditioning	Learning ability	50	M/B/F	light	3-6 months	unknown	Chaput et al., 1973
Avoidance conditioning	Excitement and emotionality	120	unkn.	inapplicable	21 days	unknown	Hammel et al., 1975
Avoidance conditioning	Learning ability/ Ability of response inhibition	18	M	inapplicable	3 weeks	low calorie/ low protein/ at. lib.	Barnes et al., 1969
Preference test	Time perception and anticipation of future events	12	F(p)	commercial pellets	8 months	restricted (unknown %)	Spinka et al., 1998
Spatial learning and memory tasks							
- Alley mazes							
Three-choice-point water-maze	Spatial learning and memory	120	unkn.	unknown	45 days	unknown	Hammel et al., 1975
Adjusted Hebb-Williams maze	Learning ability and (long-term) memory	48	B/F	commercial pellets	11 and 20 weeks	abstention 12h pre-testing	Jong et al., 2000
Spatial maze	Spatial learning and memory	27	B/F	sow and litter	5 days	at libitum	Siegford et al., 2008
Modified Morris water maze	Spatial learning and memory	27	B/F	a dry location (platform)	14 days	at libitum	Siegford et al., 2008
- Free choice mazes							
Foraging arena	Spatial learning and memory	8	M	unknown	48.06 ± 1.72 kg	80% of daily ration	Mendl et al., 1997
Eight-arm radial maze	Spatial learning and memory	10	M	sow rolls	30-35 kg	70% of daily ration	Laughling et al., 1999
Multi-room maze	Learning abilities	27	F	apple	3-4 months	unknown	Hagl et al., 2005
Eight-arm radial maze	Spatial learning and memory	20	M	sow rolls	30-35 kg	75% of daily ration	Laughlin and Mendl, 2000
Eight-arm radial maze	Spatial learning and memory	16	M	sow rolls	10-12 weeks	80% of daily ration	Laughlin and Mendl, 2004
Spatial arena	Spatial learning	84	M/F	unknown	15-17 weeks	at libitum	Sneddon et al., 2009
Cognitive holeboard	Spatial learning and memory	20	F	chocolate raisin	13 weeks	restricted (unknown %)	(Arts et al., 2009)

Task	Tested cognitive ability/ abilities	N	sex	reinforcer	age (or weight)	food restriction schedule	Author
Restricted retrieval choice test	Spatial discrimination and memory	9	F	sow rolls	28.8 kg ± 2.42	80% of daily ration	Held et al., 2005
T-Maze (delayed non-match to sample task)	Spatial learning and memory	8	B	mini-pellets in water	12-14 months	70% of daily ration	Nielsen et al., 2008
Recognition tasks							
- Object recognition							
Spontaneous object recognition	Object recognition memory	8	M	-	13 months	restricted (unknown %)	Moustgaard et al., 2002
Spontaneous object recognition	Object recognition memory	16	M	-	12-14 months	fed 2x daily	Kornum et al., 2007
Spontaneous object recognition	Object recognition memory	64	B/F	-	27 days	unknown	Gifford, 2005
(Modified) spontaneous object recognition	Object recognition memory	36	B/F	-	35 days	at libitum	Gifford et al., 2007
- Social recognition							
Social recognition based on olfactory cues	Social discrimination/ recognition learning	2	unkn.	commercial pellets	6-9 months	unknown	Meese et al., 1975
Y-maze	Social discrimination/ recognition learning	32	M	-	6-7 weeks	at libitum	Kristensen et al., 2001
Y-maze	Social discrimination/ recognition learning	12	F	raisins	6 weeks	at libitum	McLeman et al., 2005
Social recognition test	Social discrimination/ recognition learning	120-132	M/F	-	11-13 days	at libitum	De Souza et al., 2006
Observational learning							
'Informed forager' paradigm	Exploitation of knowledge of others	16	F	unknown	>29.87 kg	70% of daily ration	Held et al., 2000
Adapted Guesser-Knower experiment	Exploitation of knowledge of others	18	F	commercial pellets	juvenile	70% of daily ration	Held et al., 2001
Awareness							
Mirror Test	Object/ information use	19	M/F	food	4-8 weeks	at libitum	Broom et al., 2009

reinforcement (i.e., the number of responses needed to earn a reward increased during the course of the study) was applied for each sweetener separately. It was found that pigs were willing to work to very high break points (the point at which they were no longer willing to work for the reward) to obtain sucrose and glucose, and to high break points for saccharin (Kennedy and Baldwin. 1972). This is one of the studies (see 'Sensory capacities') confirming that pigs like sweet tastes. Sneddon et al. (2000) applied a reinforcement schedule with fixed ratio 2 and 3 (i.e., every second or third lever press was rewarded) during a leverpress experiment. Boars and gilts were housed in barren or enriched environments. No gender differences were found, but in general animals from an enriched housing environment acquired this task more rapidly.

Recently, Ferguson et al. (2009) exposed 6 female Yucatan minipigs to a food reinforced lever-pressing experiment. They applied a progressive ratio reinforcement (PR) schedule (PR1 + 2, i.e. in each successive trial, 2 more lever presses were needed to gain the reward than in the previous trial). The response rates of these prepubertal minipigs ranged between 0.48 and 1.99 lever presses per second. This means that, on this task, pigs have higher response rates than rats (which show lower response rates) but lower response rates than non-human primates (which show higher response rates). Ferguson reused the minipigs in a 'temporal response differentiation training' task, in which a reward was given when the lever was held down for a minimum of 10 and maximum of 14 s. Acquisition of this task was poor, but the researchers presumed that this was more likely the result of the apparatus and the physical response of the minipig (hooves easily slipped off of the lever) than the difficulty of the task itself. The last operant test used, involving 3 of the 6 minipigs from previous experiments, was 'incremental repeated acquisition', a progressive task in which in every trial several levers have to be pressed in a different order. Again, acquisition of this task was relatively poor, but the study lasted only for a short time and food deprivation was not applied. The design of these experiments was not optimal because the number of pigs was limited, training could not be continued long enough, and the apparatus was not adapted to pigs. Still, these studies support the notion that automated conditioning equipment can be used to present tasks of varying difficulty, which means that it is possible to compare the performance of rodents and pigs.

Apart from Skinner box-like operant lever-pressing experiments, various other positively reinforced operant conditioning tasks have been used. In a study investigating early development and later learning, Lien and Klopfer (1978) trained pigs in a discrimination reversal test. Piglets that showed a very strong preference for a particular teat (termed 'stereotyped suckling' by Lien and Klopfer) were compared with piglets that varied their suckling position. In an operant test comparing these 2 groups, hog pellets could be obtained by responding to the correct response panel on the stimulus apparatus lowered into the pen (presented together with a light cue). The apparatus was retracted after the pigs made a response. The automatic feeder was placed in another corner of the room. When the piglets had reached a preset learning criterion, they were trained on reversal of this problem (i.e., instead of responding on the side where the stimuli appeared, responding at the opposite side was rewarded). While there were minimal differences in task

acquisition between the 2 groups of piglets, reversal learning appeared to be more difficult for piglets that showed strong teat preferences before weaning. Although 'teat order' is generally considered to be stable in piglets (i.e., Fraser and Thompson (1991)), rendering the term 'stereotyped suckling' somewhat unsuitable, Lien & Klopfer showed that these learning experiments can be useful to investigate behavioral problems that are related to early development.

In 2004, Bolhuis et al. studied reversal learning in a T-maze, using pigs from 8 litters (housed in barren or enriched environments). These animals were first tested twice in a back-test (restraining a piglet for 60 s on its back) and classified as high- or low-responding according to the number of escape attempts. During training, one arm of the T- maze contained bait, but during 6 consecutive trials this arm did not contain bait, or an intramaze change was applied (placing of a novel object in the baited arm). During training trials, no housing, back-test classification, or sex differences were found. However, the reversal trials revealed low-responding piglets to perform better (i.e., they entered the new baited arm more often) than high- responding piglets. When an intra-maze change was applied, an interaction between housing and back-test classification was found: high responders from a barren environment spent more time investigating the novel object than low- responders from the same environment. However, low-responders from an enriched environment were found to be more distracted than the enriched housed high responders. Thus, although pigs learn this type of T-maze discrimination learning task relatively rapidly (i.e., the task is relatively simple), differences between groups of pigs can be detected by increasing the difficulty of the task, by introducing a reversal. Interestingly, coping style seemed to be related to performance.

Tanida et al. (1991) and Tanida and Nagano (1998) investigated whether pigs are able to discriminate between green, blue, and red, using a T-maze. Two female weanling pigs were trained to discriminate pairwise between the 3 colors. Additionally, 2 other weanlings were trained to discriminate pairwise between gray and 1 of the 3 colors (all colors had the same luminosity). All animals were able to discriminate between blue and all other colors, but not between green and red and green or red versus gray (Tanida et al. 1991). More about visual performance studies can be read in 'Sensory capacities'.

In an operant conditioning task, Tanida and Nagano (1998) trained pigs to discriminate between a familiar and an unknown handler in a Y-maze. Animals had to respond to the familiar handler by entering the arm of the maze in which that person was present. A correct choice was rewarded with chocolate raisins. To find out pigs' responses to changes in visual, auditory, and olfactory cues, hints were changed one at a time (i.e., no calling of the pig or wearing the same perfume anymore). This study demonstrated that pigs are able to discriminate between humans. Visual and auditory cues seemed to be more important than olfactory cues, but the variation in individual performance was large. In 1999, the handler discrimination experiment was repeated by Koba and Tanida, but this time visual cues seemed to be the most discriminative factor: when all handlers wore the same color clothes, only a few pigs were able to make the correct choice (Koba and Tanida. 1999).

To gain insight into human-avoidance behavior, Hemsworth et al. (1996) studied pigs' associative learning capacities in a human approach test. Three groups of sows received different types of treatment given by humans (e.g., a boar was introduced daily to the sow by a handler, or 'back pressure treatment' was applied daily by a handler, or the sow was minimally treated by a handler) during 2 estrus periods with or without the presence of food before being exposed to the test. During testing, all behaviors directed toward the human were scored. Hand-fed pigs were found to be less fearful, taking a shorter time to approach the experimenter.

The above experiments revealed that pigs can distinguish between familiar and unfamiliar persons. This knowledge could be used to improve animal welfare. However, because these experiments investigated not only on the pig's memory, but also its sensory capacities such as sight, smell, and hearing, although not distinct from each other, future studies should try to separate the different sensory cues from each other.

Croney et al. (2003) applied relatively simple operant discrimination learning tests in pigs. Four minipig boars were trained to discriminate between colors (orange or green) or olfactory stimuli (coconut or almond) to earn a food reward. An experimenter used a clicker directly after a correct choice was made, and the pig approached the experimenter to obtain its reward. Pigs could discriminate between cues, and when the task presented multiple choices simultaneously (2-10 smells or colors), pigs were still able to respond to the correct stimulus. Croney et al. suggested that the animals might have formed a general learning set that transferred across tests with varying amounts of simultaneous choices. What remains unknown from this experiment is the number of sessions (10 trials/session) needed to reach criterion of learning for each new phase in which one stimulus was added. This omission makes it hard to interpret how easily pigs learn to discriminate between 2 or more visual or olfactory stimuli.

Using a non-spatial version of the radial-arm maze, Wang and Xu (2007) studied 2 learning tests ('easy' and 'difficult') in succession. A visual cue (1 versus 3 black dots in the easy and 2 versus 3 black dots in the difficult task) was used, and the arm marked with 3 black dots hid accessible milk replacer. In both tests, 40 trials were given divided over 5 and 6 days, respectively. Memory was tested 2 days after completion of a set of trials, by presenting the same task again in one trial. Pigs were able to acquire both the easy and the difficult tasks, but criterion level was reached in fewer trials during the 'difficult' task, suggesting that previous acquisition of an 'easy' task facilitates the acquisition of a subsequent harder task. Pigs supplemented with sialic acid (possibly a conditional nutrient during rapid brain growth) acquired both tasks in fewer trials; however, it cannot be ruled out other domains, such as motivation, were affected. Memory was also positively influenced by sialic acid (2 days after completion of a set of learning trials, a single memory test of one trial only was performed), with the sialic acid-supplemented animals having higher scores than the controls. These results show that this visual discrimination task could be used to study the effects of putative cognition enhancers.

Moustgaard et al. (2004) trained Göttingen minipigs on black–white (visual) and right–left (spatial) discrimination by teaching them to put their nose in a response hole for a food reward. After acquisition of the discrimination task, it was investigated whether the pigs paid attention to particular stimulus dimensions by applying an extra-dimensional shift procedure (changing the stimulus dimension from visual to spatial or the other way around). Stimulus dimensions were not found to be important and nor was the pig's sex, with boars and sows reaching the same level of performance. More recently (2005), Moustgaard et al. trained minipigs in a right–left discrimination task followed by a go/no-go task. To acquire the left–right discrimination task, animals were trained to respond in the left hole when both holes turned black and to respond in the right hole when both holes turned white. During the go/nogo task, pigs were rewarded with food if they responded when both holes turned blue and were mildly 'punished' (20 s of darkness) if they responded when both holes turned red. All pigs reached the criterion of [90% correct choices per session for the right–left discrimination task and nearly all reached this level for the go/no-go task.

Moustgaard et al. (2005) provided information about the time needed to train pigs in their visual and spatial discrimination tasks, information that unfortunately is missing in many other studies. Two out of 16 minipigs were not able to reach criterion of learning during the 1st step of the shaping phase (putting the snout in a response hole), and 2–15 sessions of 40 trials each were needed for the next step of the shaping phase. Criterion during the last step of the shaping phase was reached in 2–11 sessions. During the actual discrimination experiment, it took the animals 1–20 sessions of 20 trials each to learn the discrimination or reversal. This schedule for extensive training and pretraining shows that discrimination experiments with pigs can be very time-consuming. Moreover, the behavior needed to perform this task might not be part of the pig's natural behavioral repertoire, which may mean that the pig cannot 'learn' the task or that it becomes demotivated.

Recently, Friess et al. (2007) examined the principle of operant learning in a 'food cover task'. Female piglets were trained to remove a plastic cover from a food dish to gain access to the hidden food within 20 s. In a 'glass barrier task', piglets were trained to move around a transparent barrier in order to gain access to the food. These tests were part of a test battery used to obtain information about discrimination learning abilities after mild or moderate brain injury, which was induced by rapid axial head rotation. Both the brain-injured piglets and the control piglets performed the tasks at the same speed. Unlike other tests of the test battery (i.e., neurobehavioral tests such as beam walking), these learning and memory tests apparently fail to detect (subtle) deficits.

Aversive learning

During the late 1960s, Kratzer (1969) studied shockmotivated avoidance learning in Duroc and Hampshire pigs, using a shuttle box. The shock was delivered via a girth around the chest if the pig did not cross a wooden barrier when an avoidance signal (buzzer) had sounded. Tests were performed with pigs up to 160 days of age. Younger, approximately 20-day-old, pigs showed better avoidance learning (crossing the barrier) than older pigs, and heavier pigs were better learners than

lighter pigs of the same age. Kratzer hypothesized that weight might be positively correlated with factors that increase learning performance, such as physiological maturity and general health. Results also clearly showed breed differences in learning, with Duroc pigs achieving higher levels of avoidance learning than Hampshire pigs, regardless of age. Because birth weight differences between low and normal-birth-weight piglets are known to be lasting (Rehfeldt and Kuhn. 2006), one could speculate that birth weight is correlated with learning performance.

Chaput et al. (1973) trained 3- to 6-month-old pigs in a one-way shock-motivated avoidance shuttle box task in which a telephone buzzer was used as auditory CS. After the buzzer went off, pigs could avoid a subtetanizing shock by moving from a darkened to an illuminated chamber. If the pig did not cross into the illuminated chamber before the US, they were given an electric shock (max. duration 93.9 s). The average level of shock avoidance was very high. In their comparison of shuttle box learning and water maze learning (an aversively motivated spatial discrimination task), Hammell et al. (1975) found that the performance of pigs on the 2 tasks was uncorrelated and concluded that these 2 tasks tap different behavioral domains (e.g., motivation, sensory requirements).

Barnes et al. (1969) exposed pigs to a conditioned avoidance procedure in a large quadrangular arena with several hurdles. Shocks could be avoided by jumping a hurdle when a CS was presented (clicking signals). After 3 training sessions, an extinction session was run (i.e., the CS was no longer followed by a shock). Pigs that were malnourished early in life displayed higher levels of excitement (ethogram to score excitement was not described) in the extinction sessions and were unable to inhibit responses when the CS was presented. This operant task shows how the influence of specific (negative) early life conditions can be studied in an aversively motivated operant learning task.

Time perception and anticipation of future events were measured by Spinka et al. (1998) in a preference test. Pregnant sows were trained to enter 1 of 2 rooms, each containing several feeding crates. The rooms could be reached by turning left or right from a corridor. After the sow entered a crate, it received food and the crate closed automatically. The crate opened automatically again after 30 min (left room) or 240 min (right room). After a training period (i.e., in which sows were only allowed to enter one of the rooms), most sows entered the left room with the short confinement crate, which suggests that pigs can perceive time.

Spatial learning

Alley mazes: The aversively motivated three-choice point water maze (a rectangle tub in which pigs have to swim from one side to the other with three left–right choice points in-between) is an example of a spatial learning task in an ‘alley’ type maze. Using this water maze, Hammell et al. (1975) found that pigs (n = 120) could readily learn alternating (exit not visible, pig swims around barriers) but not non-alternating (exit visible straight ahead, pig swims through openings in barriers) swimming patterns. de Jong et al. (2000) studied learning and long-term memory in a dry maze with different configurations, based on the Hebb–Williams maze. Piglets acquired the task at the age of 11 weeks, and

retention was tested 9 weeks later. The goal of this study was to investigate whether housing in barren or enriched environments affected learning and memory. Piglets from both groups learned the maze configurations quickly, but the piglets raised in a barren environment made significantly more line crossings and had a longer latency to reach the food when the previously learned maze configuration was repeated in the retention test. De Jong et al. concluded that piglets raised in a barren environment had an impaired long-term memory compared with piglets housed in an enriched environment. The authors showed that it is possible to study postnatal influences on memory in a relatively simple maze test.

A more recent maze test designed by Siegford et al. (2008) was used to assess the effects of maze training on weaning stress in 5-day-old piglets. The maze used can be classified as an 'alley' maze, but increasingly complex variants were used. Here, instead of food, the reward was being returned to the home pen with the sow and littermates. Each piglet was randomly assigned to 1 of 3 different groups ('maze task', 'short isolation control', and 'control with sow'). Piglets that were maze trained, i.e. were exposed to cognitive challenges, showed a decreased fear of unfamiliar persons after weaning compared with control piglets housed under the same conditions. The authors also investigated whether early cognitive experiences influence learning ability. This was tested in a 'watermaze spatial memory task' (38–39°C heated pool with a diameter of 3.6 m) similar to the Morris Water Maze (Morris. 1984). The outcomes of the male piglets previously trained in the first maze were notable: they escaped onto the platform faster than did piglets that were previously exposed to short-lasting social isolation. However, no differences were found between 'control' and 'maze-tested' animals, and therefore these results cannot be indisputably ascribed to the prior cognitive experience of the piglets. More research is needed to show whether cognitive enrichment at a very young age influences cognitive performance later in life.

Free-choice mazes: Most spatial learning and memory studies in pigs have used 'free-choice mazes'. For example, Mendl et al. (1997) used a foraging arena to study spatial memory and its susceptibility to disruption by environmental stimuli. Ten identical food troughs placed against the walls of the arena were covered by panels and could not be seen by the pigs. In this repeated acquisition paradigm, one trough was baited, and pigs were allowed to search for it during a sample trial. During choice trials (i.e., relocation visits), pigs found their food in fewer visits than expected by chance, but disturbances during the inter-trial interval (e.g., isolation in a novel environment) resulted in more errors made during choice trials. This indicates that pigs isolated in a novel environment are susceptible to mild disruptions of spatial memory. This task seems to be suitable to measure the influence on spatial memory of disturbances during inter-trial intervals. A possible element of the task that might need some consideration is the number of food troughs. Yerkes and Coburn (1915) applied a similar task using 9 parallel entry doors. Their results showed that pigs have difficulties discriminating between doors. They performed best on the left, right, and middle doors, but had difficulties with discriminating or remembering the in-between doors. This might also be true for the radial-arm maze experiment performed by Laughlin et al. (1999). During the first experiment, the authors baited 4 out of 8 arms, and pigs were allowed to

locate and eat the food freely. Spatial memory was tested after a retention interval (10 min) with or without a disturbance factor (e.g., weighing in a crate). Pigs that were weighed took longer to find the food reward, showing that environmental stimuli can disrupt memory, in this case for baited food sites (Laughlin et al. 1999).

Recently, efforts have been made to develop learning and memory tasks for pigs in which several factors can be controlled and measured experimentally, comparable to what is currently standard practice for other species. For example, Hagl et al. (2005) designed a multi-room maze to study possible subtle learning impairments in pigs that suffered from induced hypothermic circulatory arrest (HCA). Six out of 8 rooms in the maze were baited with apple. During each trial, an animal was only allowed to visit one of the rooms (other doors closed directly after entry of this one room). Between trials, the animals waited in a holding area for 30 s. Scores (i.e., decrease in the number of entries of unbaited chambers) improved over a 12-day period. A daily training session ended when all baited chambers had been visited or after 20 trials, whichever event occurred first. For the second problem again 12 days), only the left 4 or the right 4 chambers were baited. Every day, during the second half of the trials, the baited rooms switched sides. This second problem was designed to increase the level of difficulty, to detect subtle differences in learning and memory. Learning and memory were similar in HCA animals and healthy subjects in the first task, but the performance of the HCA animals was impaired in the second task. The outcomes of this study clearly underline the importance of considering the sensitivity of a test design—too simple designs might yield false-negative results.

Laughlin and Mendl (2000) studied win-shift/win-stay strategies in pigs in a radial-arm maze. Four arms were baited, and these arms were rebaited for pigs assigned to the ‘stay’ strategy during the recall trial. The reward for the ‘shift’ pigs could be found in the 4 previously unbaited arms. The results showed that pigs are capable of using both a win-shift and a win-stay strategy, but that the task is performed faster and with a higher degree of accuracy after training in the win-shift task. When the costs to obtain food in the baited arm in the radial-arm maze were increased (i.e., more effort needed to reach the reinforcer, by placing a rope in the way) during the sampling trial in the win-stay task, the number of errors made during choice trials significantly decreased, possibly because pigs paid more attention to the baited arm.

Sneddon et al. (2000) tested pigs reared in barren and enriched environments in a spatial foraging arena. This arena was divided into 12 squares, with 7 of these squares fitted with food bowls. Only one bowl contained a food reward. The piglets (both sexes) raised in the enriched environment found the baited bowl significantly faster than the piglets raised in the barren environment. This is one of several experiments showing that an enriched rearing environment improves learning and memory.

In a spatial holeboard discrimination task, Arts et al. (2009) showed that mild mixing stress did not influence pigs’ performance. Mixing stress was induced by housing a pig in a new pen together with an unfamiliar individual 1–4 h before

trials 1, 5, and 8 (13 trials in total). The test arena in which 16 buckets were symmetrically placed, measured 8 9 7.6 m. One entry door allowed access to the arena. After a training period, testing (consisting of 3 test phases with a total of 25, 13 and 13 trials, respectively) started. Each pig (n = 20, Finish Landrace x York F1) received its own configuration of 4 baited (chocolate covered raisins) holes. The configuration for each individual changed per test phase. Without food deprivation, pigs rapidly learned to search and collect baits and thus acquired the tasks. Performance (WM and RM) improved over test phases, and it was concluded that the animals 'learned to learn'.

A somewhat different but comparable task used by Held et al. (2005) showed the relevance of utilizing spatial tasks when investigating pigs' abilities to discriminate between food of different value. In a restricted retrieval choice test, Held et al. (2005) investigated whether domestic pigs could remember baited areas and differences in the amount and quality of the baits. In this version of a spatial memory task, 2 out of 8 possible food sites contained bait. The amount of food (8 versus 3 sow roll pieces) together with the addition of an obstacle (a brick) determined the relative value of each baited location. After a training period, pigs were only allowed to visit one of the food sites. The outcomes suggest that juvenile female pigs can discriminate between food sites of different value and overall choose for the site with the largest bait.

Recently, Nielsen et al. (2009) trained pigs in a reinforced T-maze alternation task to find a reward in one arm during the first trial and in the opposite arm in the second trial. The number of correct choices during several trials is a measure of spatial short-term memory. Pigs were able to perform this task with delay intervals of 60, 300, and 900 s. When treated with scopolamine (an anti-cholinergic drug that causes memory dysfunction; 0.40 mg/kg^{-1} intramuscularly), the number of errors increased for all time intervals, and the speed of task performance decreased. By administering scopolamine after untreated animals had performed the task, Nielsen et al. clearly showed that this task measures memory performance, and their findings can be seen as a step forward in the validation of such tasks.

Recognition tasks

Tests that assess the recognition abilities of pigs can be subdivided into the recognition of objects, conspecifics (social recognition), and humans.

Object recognition

Moustgaard et al. (2002) demonstrated that Göttingen minipig boars are able to acquire the object recognition test (ORT). Non-castrated boars were tested because they were expected to be more explorative than sows. Pigs were first habituated to a test arena, and then to an arena containing 2 identical objects. One hour later, after one of these objects had been replaced by a non-familiar object, the pigs entered the arena. The boars investigated the novel object, but there was substantial variation in how long they investigated it. It was concluded that memory for objects lasted at least 1 h. Kornum et al. (2007) found that pigs could discriminate between familiar and novel objects, as evidenced by a longer time spent investigating a novel object during the ORT, but only when the retention interval was shorter

than 1 h. The authors tested twelve different sets of objects and found that the time spent investigating the various objects was different, possibly due to differences in object preference.

In contrast, Gifford et al. reported that pigs failed to display novelty preference at any delay interval in the ORT, possibly due to the length of the exposure phase and the location where pigs were exposed to the familiar object. Unlike tests with human infants and rodents, pigs were exposed to the object in their home cage with littermates, instead of being alone. Gifford also suggested the possibility that these results were due to the lack of preference for an unfamiliar over a familiar object, even though the animal recognized the familiar object (Gifford. 2005, Gifford et al. 2007). The few object recognition studies involving pigs published to date have yielded contradictory results opposite to those reported in rodent studies (which show that the animals have a stable preference for investigating a novel object (Ennaceur and Delacour. 1988)). Consequently, it still remains to be demonstrated that the ORT is useful to test object recognition memory in pigs.

Recognition of conspecifics

Meese et al. (1975) found that pigs could distinguish between urine samples from conspecifics. In the experimental set-up, gilts had to respond, by means of panel switching, to the correct odor stimulus in order to gain a food reward. Mendl et al. (2002) presented urine samples of unfamiliar conspecifics to 22 female Large White Landrace pigs to investigate whether they could discriminate between urinary odors of animals of similar age. In order to study this, a habituation–dishabituation procedure was applied in a control and a discrimination group. An animal was presented with a fresh urine sample from another animal for 2 min, followed by an interexposure interval of 15 min. After this interval, the sample was presented again for 2 min. After another 15-min interval, the sample was presented again for 2 min. The duration of urine sample investigation was recorded. Shorter durations (habituation) were expected when a sample from the same pig was presented a second time and longer durations (dishabituation) were expected when a urine sample from a different pig was presented. The discrimination group was presented with 2 different samples from one individual and one sample from a different individual. In this experiment, which is based on investigatory behavior, Mendl et al. (2002) showed that 10-week-old gilts are able to discriminate between urine samples from conspecifics. The habituation–dishabituation procedure was successful in showing stimulus discrimination in pigs, although exploration time appeared to be the only useful measure (Mendl et al. 2002).

The ability of pigs to recognize familiar conspecifics was studied by Kristensen et al. (2001). Using a Y-maze with a familiar and an unfamiliar stimulus pig behind doors that allowed tactile, visual, and olfactory contact or olfactory contact only, the authors found that juvenile animals responded well to (familiar) social cues and concluded that pigs are able to discriminate between familiar and unfamiliar conspecifics. The variables studied were time spent in zones in close proximity to one of the conspecifics and the number of entries to those zones. This study suggests that pigs are more motivated to visit sites containing several social cues

(i.e., tactile, olfactory, and visual) rather than only one such clue (olfactory). In 2005, McLeman et al. confirmed the finding that juvenile pigs could successfully discriminate between familiar littermates and unfamiliar individuals in a Y-maze. Pigs spent more time in close physical proximity to the familiar pig compared with the unfamiliar pig. de Souza et al. (2006) also found that neonatal piglets had good short- and long-term social recognition performance and that social memory was not influenced by minor changes to the environment (relocation of sow and litter in a new pen).

The above-described studies all showed that pigs prefer staying in close proximity to familiar conspecifics if given the choice and thus confirm that pigs are able to discriminate between conspecifics. This basic knowledge about the species could be used when designing tasks to provide greater insight into social recognition in pigs (e.g., number of conspecifics recognized, long- and short-term social memory).

McLeman et al. (2008) continued their conspecific-discrimination studies using 12 Landrace X Large White X Duroc pigs. They used a Y-maze to show that pigs are able to discriminate between individual group members, using either bimodal or unimodal cues. The end of each arm of the Y-maze contained a rewarded and unrewarded stimulus pig. In this closed maze controlled for olfactory, visual, and auditory cues, pigs first had to learn a bimodal task. Animals were trained to discriminate between a pair of familiar, but unrelated littermates by using 2 of 3 sensory modalities (audition, olfaction, and vision). After the pig reached the learning criterion (3 consecutive sessions 8/10 correct choices), it was transferred to a unimodal task in which discrimination was based on only 1 of the 3 modalities. Daily sessions of 10 consecutive trials were given, and approaching the correct stimulus pig was rewarded with raisins. Although 4 animals did not reach the learning criterion in the bimodal test and 2 of the 8 animals successful in the bimodal test did not reach the criterion in the unimodal test, McLeman et al. showed that pigs are able to discriminate between related group members when only 1 or 2 sensory modalities are available. The closed and controlled environment of this Y-maze provides opportunities for further studies on social discrimination and mental representations without using invasive techniques (McLeman et al. 2008).

Observational learning

Observational learning-like studies have investigated socially cued behavior in pigs. However, results do not provide clear evidence that pigs are able to imitate a conspecifics' behavior or exploit its knowledge. Baldwin (1979) observed the social behavior of 2 or 3 individuals at a time in a lever-press room. These individuals could be familiar or unfamiliar, trained to press the lever, or untrained. The authors reported that subordinate pigs pressed the lever at a low frequency and that observational learning did not occur. Dominant pigs were found to do most of the lever pressing, but lever-press frequency declined in all pigs with increasing test duration, possibly because of satiation effects (Baldwin. 1979). However, it can be questioned whether observational learning can be properly investigated in this experimental set-up. Even though a pig might have learned from its conspecifics how to earn rewards, its hierarchical position within the group might prevent it from pressing the lever.

Held et al. (2000) studied this phenomenon using the 'informed forager' paradigm. In a spatial arena (see 'spatial memory'), food was hidden in 1 of 8 buckets. A pig that had been trained to find the food entered the arena together with a heavier and non-trained pig. The results appeared to show that the non-trained pig followed the example of the trained pig rather than randomly investigating the buckets. Held described this behavior as the 'exploitation of knowledge of an individual by another pig'. Held et al. Investigated observational learning further in 2001, testing the hypothesis that pigs can discriminate between companions who can see where food is hidden and companions who cannot. Results provided weak evidence for the notion that pigs have visual perspective taking abilities, i.e., the ability to appreciate that others can or cannot see (Held et al. 2001). Because little is known about this type of cognitive ability in pigs and the tests used did not provide unambiguous evidence, further research is needed.

Awareness

Very recently, Broom et al. (2009) assessed the ability of 4–6-week-old pigs to use information acquired with a mirror to locate a reward in a food bowl. Pairs of pigs from one group (n = 8) were placed in a pen with a mirror for 5 h and a pair of pigs from the control group (n = 11) were placed in a standard pen. Thereafter, a 'mirror test' was performed. Each piglet was individually released in a room with a mirror. A barrier, placed against the mirror at an angle of 90°, divided the first two-thirds of the room. The piglet entered the room at the back on the right side. From there, it was able to see a food bowl in the mirror, placed on the other, not directly visible, side of the barrier. Piglets were allowed to walk around the barrier and the mirror. Nine of eleven control pigs first approached the mirror and then walked behind it; however, 7 of 8 piglets with mirror experience looked at the mirror, saw the food bowl, and went to the other side of the barrier to obtain the food. The mirror-experienced pigs were presented with the same setup again but with the mirror replaced by a wire mesh. The food bowl was placed behind the mesh at the same location where it had been visible before in the mirror. Of the 8 animals, 6 went to the area with the food bowl, behind the mesh. This experiment showed that piglets are able to observe and remember features of its surrounding and can act accordingly. To turn away from the mirror with the image of the food and to go around the barrier to get to the food requires piglets to have a mental map of the environment and awareness that it can access the food reward. By excluding other potential cues, such as smell and area preference, Broom et al. were able to show that pigs can learn how a mirror functions and how to exploit this knowledge. However, findings do not necessarily imply that the pigs recognized themselves, but this ability is the first step in the process of self-recognition (Macellini et al. 2010). Such studies have not yet been performed with pigs.

Discussion

The pig in cognitive research: a twofold goal

Pigs appear to be a very suitable and promising animal species for use in biomedical research investigating learning and memory (de Groot et al. 2005, Lind et al. 2007, Nunoya et al. 2007, Vodicka et al. 2005), and a number of behavioral cognitive tasks have been developed using these animals (Chaput et al. 1973, Larsen and Rolin. 2004, Lind et al. 2004, Mikkelsen et al. 1999, Moustgaard et al.

2005, Nielsen et al. 2009, Schook et al. 2005). Pharmacological or experimental manipulation of brain structures has been performed in an attempt to modulate the pigs' learning or memory abilities but, compared with the number of rodent studies, relatively few such studies have been performed. There is an urgent need to standardize and (pharmacologically) validate learning and memory tests for pigs.

Information gathered from studies of animal brains influences how we think about species with specific cognitive abilities (Broom and Zanella. 2004). These studies also tell us something about the way an animal perceives its environment (Broom and Zanella. 2004) and about the complexity of concepts that animals have (Broom. 2010). Cognition studies can directly or indirectly contribute to improving animal welfare. For example, it is now known that pigs can recognize their handlers, have preferences, and benefit from environmental enrichment. These aspects should be considered when looking at ways to improve their welfare (Manteuffel et al. 2009). The general public is becoming increasing alert to farm animal welfare, and information and decisions about animal welfare need to be evidence based. Studies of both a fundamental (e.g., pigs capabilities in general) and applied (e.g., the influence of specific treatments on cognitive development) nature are relevant in this context. Although the aims of biomedical and animal welfare scientists are different, behavioral test paradigms are relevant to both fields of research, providing complementary information.

The need for validation and replication of paradigms

This review of the literature on cognitive research in pigs has highlighted deficiencies in both lines of research. More needs to be learned about emotional factors influencing learning in pigs (Lind and Moustgaard. 2005), the relation between stress and cognitive function (Mendl. 1999), pigs' discriminatory abilities (McLeman et al. 2005), comparison of the cognitive abilities of pigs and other model species, such as mouse, rat, and monkey (Moustgaard et al. 2005), pigs' perception of time (Spinka et al. 1998), memory for objects (Gifford et al. 2007), social and observational learning (Held et al. 2000, Held et al. 2001), cognitive abilities related to foraging behavior (Puppe et al. 2007), and cognitive abilities (Ferguson et al. 2009).

While our knowledge of pigs is increasing, there is a need for validated and translational behavioral paradigms (Kornum et al. 2007). The broad variety of experimental findings published in recent years has highlighted the learning abilities of pigs and has indicated which test paradigms might be suitable for this species (e.g. Ferguson et al. (2009) and Nielsen et al. (2009)). However, the drawback of the great diversity of paradigms used in pig research is that most studies have not (yet) been replicated. Consequently, little is known about the reproducibility and generalizability of results. To date, there is insufficient knowledge to consider pigs as a standard model for biomedical studies of learning and memory. There is always the danger that a number of these studies have yielded idiosyncratic outcomes (van der Staay. 2006), and thus it is important to replicate the results of earlier studies, to consolidate the knowledge base (Muma. 1993, van der Staay. 2006, van der Staay et al. 2009, van der Staay et al. 2010).

Table 2. Overview of cognitive tasks applied in pig research and their opportunities for implementation in the field of animal welfare and biomedical research. Criteria are based on chapter 1.3 (Implementation of cognitive tasks). + indicates a positive expectancy for this criteria in a particular test category, based on acquired results or analysis of the test construction. +- indicates that the expectancy might be promising, based on comparable tests applied in other species or analysis of the test construction. – indicates a negative expectancy for this criteria in a particular test category based on acquired results or analysis of the test construction. Due to the multiplicity of tests applied within pig research so far, the categorization made here is a broad outline and some types of tests are be piled up to keep this table specific and to secure a convenient arrangement.

Criteria: **Test category:**	**Unimpaired animals should be able to acquire task**	**Allow for detailed behavioural analysis**	**Stress-free**	**Tap ecologically relevant behaviours**	**Standardization**	**Automation**	**Allow investigation of developmental effects**	**Complexity and sensitivity**
Classical tasks								
Conditioning tasks *appetitive*	+	-	+	+-	+	+	-	-
Conditioning tasks *aversive*	+	-	-	-	+	+	-	-
Operant tasks								
Lever pressing tasks	+-	-	+	-	+	+	-	+
Discrimination tasks (two choices)	+	-	+	-	+-	+-	-	+-
Discrimination tasks (multiple choices)	+	-	+	-	+-	+-	-	+
Barrier tasks	+	+-	+	+-	-	-	+-	+-
Avoidance tasks	+	-	-	-	+	+	+-	-
Choice tasks	+	-	+-	-	+	+	+-	+
Spatial tasks								
Water mazes	+	-	-	-	+	+-	-	-
Spatial arena's	+	+	+	+-	+	+	+	+
Multi-access mazes	+	+-	+	+-	+	+	+	+
Choice tasks	+	+-	+	+	+	+	-	-
Recognition tasks								
ORT	+	+	+	+-	+-	-	+	+-
Y-mazes	+	+-	+	+-	+-	-	-	+
Social tasks	+	+-	+-	+	+-	-	-	+-
Awareness tasks								
Mirror test	+	+-	+	+	-	-	+	+

Suitability of specific tasks

On the basis of this review, we can identify which tests may be appropriate for specific research goals. Some of the tests used to study the cognitive abilities of pigs are suitable for investigating multiple cognitive abilities, while others are only useful for investigating one aspect (as summarized in Table 1). If we also

take into account the essential criteria for a behavioral test for pigs mentioned earlier (see 'Implementation of cognitive tasks'), we can consider what type of tests will be most promising for specific research goals (see the overview in Table 2).

Promising advances have been made in the automation, standardization, and complexity of operant tasks for pigs. The tasks designed by Friess et al. (2007) to measure learning after (mild) brain injury seem too simple to detect (subtle) differences, and many tasks would benefit from defining the optimal range of difficulty. The operant minipig tasks used by Ferguson et al. (2009) were relatively complex and automated, but of short duration. If these tasks could be repeated or extended, it might be possible to establish the reasons for their poor performance (e.g., level of cognitive or physical difficulty, motivation, time span). This could then lead to their optimization, e.g. by increasing the level of sensitivity or complexity or by aiming at a more species-specific design, and ultimately to their standardization. Moreover, sorting out the causes of poor performance would also lead to better founded conclusions concerning between-species comparisons (Ferguson's progressive ratio lever-pressing experiment). Operant tasks like those of Moustgaard et al. (2004 and 2005) are believed to be relatively complex cognitive tasks that are potentially useful for investigating brain function in pig models of human brain disorders. Repeatedly applying tasks of increasing difficulty or complexity to define the optimal level of difficulty would provide knowledge about the range and limits of the cognitive abilities of pigs, knowledge that could be used to develop standardized tests of brain function in this species. However, what all these operant tests will always lack is the opportunity to tap different relevant natural behaviors of pigs.

Depending on the question to be answered, a spatial task might come closer to fulfilling the criteria listed in the Introduction. Free-choice mazes such as the eight-arm radial maze (Laughlin et al. 1999, Laughlin and Mendl. 2000, Laughlin and Mendl. 2004) and the spatial or foraging arena (Mendl et al. 1997, Sneddon et al. 2000) appear to be suitable for studying (spatial) learning as well as memory. Arts et al. (2009) also clearly showed the advantages of the holeboard task for investigating the influence of rearing or housing conditions on cognitive performance. Water mazes (situated somewhere between alley and free-choice mazes) for pigs (Siegford et al. 2008, Hammell et al. 1975) provide measures of both learning and memory. However, this type of 'simple' test has a disadvantage. A water maze for pigs, which are fast growing and large, would have to be adjustable in size to be suitable for testing animals of different age and size. Moreover, while the effect of swimming on the pigs' emotional state is unknown (see 'Stress and cognitive functioning'), it is likely that swimming tasks cause stress and therefore such tests are less suitable.

The freedom of movement and choice a pig encounters in free-choice tests such as a spatial arena (Sneddon et al. 2000) might mimic its foraging behavior and make it possible to measure several behavioral domains (e.g., cognitive, sensory, motor domains). Minor adaptations might make these tests suitable for measuring other (cognitive) domains such as discrimination learning

(spatial, visual, or olfactory learning of 2 or multiple objects or individuals), problem-solving skills, or motivation. Even observational learning paradigms have been tested in a spatial arena-like apparatus (Held et al. 2000). It is important not to use tasks that are too simple or too difficult, because otherwise study outcomes might be false positive or negative (like the outcomes in Hagl et al. 'first problem' (see Hagl et al. (2005)).

Y-mazes have proven effective for studying mainly social discrimination or recognition learning (McLeman et al. 2005, Koba and Tanida. 1999, Kristensen et al. 2001, Tanida and Nagano. 1998). These tests, which make use of a relatively simple apparatus, should be used more often to study the sensory capacities of pigs, about which our knowledge is relatively limited at the moment. McLeman et al. (2008) also showed that the Y-maze can be adapted to a more automated and controllable apparatus in which auditory, olfactory, and visual capacities can be tested in combination or separately. In contrast, the ORT test has proven relevance in rodents but not in pigs. In theory, this test could be promising and potentially fulfils several of the criteria mentioned in Table 2. The main question to be solved here is whether pigs show a preference for investigating an unfamiliar object when it is presented together with a familiar one.

The latest development in cognitive pig research is related to the study of 'animal consciousnesses'. To our knowledge, only one study has investigated this. Broom et al. (2009) applied a test with a mirror (not to be confused with the self-awareness Mirror Test of Gallup (1970)) to assess awareness in pigs. The outcomes of this study support the notion that pigs are able to obtain and use information from an object (mirror), and thus this mirror task may prove a valuable task to assess the higher cognitive abilities of pigs.

In conclusion, when looking at the criteria outlined for cognitive tests for pigs to obtain the preferred level of reliability and validity, simple tasks such as the Y-maze could be appropriate for some purposes, but free-choice tasks, and especially spatial free-choice tasks, are the most promising tests. These tasks can be stress-free provided that animals receive a long-enough habituation period. Automation is possible, complexity can be increased or decreased, and animals are able to show a wide range of species-relevant behaviors.

Cognitive research in pigs: prerequisites

Stress and cognitive functioning

Performing under stress or arousal is known to influence or even impair memory (Schwabe and Wolf. 2010) and to disrupt cognitive processes (Mendl. 1999), although the effect seems to be task specific. Performance on appetitively motivated spatial tasks, such as the holeboard task, may be negatively influenced by chronic stress. Studies with rodents suggest that chronic stress impairs memory performance in spatial tasks such as the holeboard or radial-arm maze, whereas learning in spatial tasks that evoke moderate to high levels of arousal (e.g., water mazes) seems to be unaffected or is even facilitated by chronic stress (see review of Conrad. (2010)). Several studies have used electrical shocks as stimulus in aversive learning tasks. The animal's response to the CS is

believed to be motivated by aversion of the shock caused by pain and/or fear. Since pain and fear are associated with stress, the use of this type of reinforcer is not recommended when studying learning and memory in pigs, except in the case of studies designed to assess learning during stressful circumstances. As stress might adversely affect results, caution is warranted if it is not known whether a certain procedure or reinforcer evokes stress. The pigs in the experiment of Spinka et al. (1998) were free to choose for short or long confinement during testing, but despite the presence of food reward, confinement still is a negative reinforcer. Thus, there is a probability that stress occurs due to negative reinforcement.

This is also true for water mazes. Although little is known about whether pigs find swimming pleasant or unpleasant, they are able to swim (Albarella et al. 2007, Bennett. 1970). However, they probably do not swim often, and therefore swimming might not be the best behavior to choose for using in learning and memory tasks. Using conspecifics as reinforcing stimuli could cause stress in piglets if they are removed from the sow and their littermates. This is what Siegford et al. (2008) did in their study. In this specific case, stress might not only have influenced learning performance, but might also have interfered with the original research question (does early cognitive performance reduce stress during weaning) because it is uncertain whether weaning stress was reduced because of the piglets' prior cognitive experience or their prior exposure to (a) stressful situation(s).

Even less is known about the influence of positive arousal (e.g., anticipation of reward) on learning and memory performance in animals. Positive arousal has been found to influence performance in humans, and probably also does so in pigs and other animals. A positive mood state can enhance cognition in humans (Ashby et al. 1999), and the mood state at the time of information retrieval influences performance. Emotional information is remembered better when mood at the time of retrieval matches the information to be retrieved (positive mood, positive material; Lewis et al. (2005)) Thus, it would be preferable to prevent negative as well as positive stress and arousal before and during testing as much as possible.

Versatility of tasks

Carefully designed reliable equipment that can be used in several tasks would provide a good basis for gathering basic, factual, and replicable results. The larger size of pigs means that test equipment will be more expensive than for rodents, and for this reason it should be appropriate for testing multiple variables and hypotheses. The apparatus should be designed in such a way that animals of different ages, breeds, sizes, and sex can be tested without evoking stress (Siswanto et al. 2008) and should be based on the pig's natural abilities.

Translatability of results

The translatability of findings from pigs to humans and other species is an important consideration when developing behavioral cognitive tests suitable for pigs. The physiology of pigs resembles that of humans, which enhances the translatability of data to humans. A paradigm that can be used for animals and humans alike is expected to promote translational value. Technological advances have led to the development of virtual versions of animal-based tasks

for use in human research, and spatial tasks in particular have made comparisons and translatability between animal and human studies easier. Examples of virtual reality spatial tasks for which analogs have been designed for humans and other animal species are the cognitive holeboard (Cánovas et al. 2008), the Morris Water Maze (Astur et al. 2002, Bartsch et al. 2010) and various spatial mazes (Grön et al. 2000, Kahana et al. 1999). Because genetically highly homogenous animals are used in most studies with rodents as subjects, whereas humans are highly heterogeneous, Hoyte et al. (2004) suggested that interventions should have demonstrated effectiveness in 2 species, in order to improve the translatability of findings. Pigs could be one of the species tested.

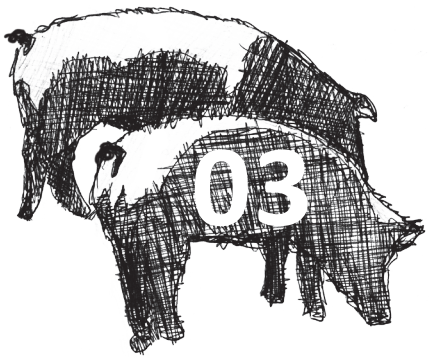
Factual knowledge about the species

A large amount of research has been performed on learning and memory in pigs. In particular, pig models are expected to have a higher translational value than commonly used rodent models. There are, however, a number of gaps in our knowledge about pigs that need to be closed. For example, little is known about the sensory capacities of pigs in general. Furthermore, there is a lack of replicated experimental findings and a lack of studies trying to optimize experimental approaches with pigs as subjects. Yet reliable equipment and validated test systems are needed to enable biomedical researchers and welfare specialists to study all aspects of learning and memory in this species. Such knowledge is a prerequisite for developing and validating pig models and for translating findings to management systems that improve pig welfare under production conditions.

Conclusion

This article has reviewed behavioral test paradigms that may contribute to biomedical research and pig welfare; however, systematic research is lacking. A critical point when designing tasks is that they should reflect the range of natural abilities of a species. To this end, further research into the sensory and motor abilities of pigs is urgently needed.

Several types of tests have proven useful. Simple two choice mazes (mainly Y-mazes) are suited to investigate social discrimination and recognition, and sensory capacities. Operant (lever-pressing like) tests meet several of the criteria that tasks for testing cognition in pigs should fulfill. In particular, they can easily be automated and standardized. Free-choice spatial tests seem to be especially promising. In contrast to operant tasks, they are able to measure several behavioral domains simultaneously, and various paradigms have successfully been developed. While these tests appear to be promising instruments to evaluate the cognitive abilities of pigs, validation studies are still lacking. The growing interest in pig models for cognitive research and the need to improve animal welfare might provide the impetus needed to lift cognitive pig research to a higher level.



Juvenile pigs use simple geometric 2D shapes but not portrait photographs of conspecifics as visual discriminative stimuli

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Abstract

Several animals living in social groups, such as monkeys, cows and sheep, have been shown to use facial discrimination for social recognition. Whether pigs can discriminate between faces of conspecifics purely based on visual stimuli provided by 2D portrait photographs, has not yet been investigated. Therefore, in this study piglets with a large birth weight range were trained in a visual discrimination task. Piglets were derived from different litters; from each litter same sex siblings with a low (LBW) and normal-birth-weight (NBW) were selected. With this setup it could be clarified whether pigs are able to discriminate between 2D photographs of conspecifics, and if LBW animals have more difficulty doing so than NBW siblings.

Because pigs learn visual discrimination tasks slowly, we started with a simple discrimination task involving one of two geometric black-and- white stimuli, followed by a simultaneous discrimination task in which one of the black-and-white stimuli served as S^{plus}, the other as S^{minus}, followed by a reversal. Pigs needed on average 337 trials in the simple and 98 trials in the simultaneous discrimination task to reach criterion. Only ⅓ of all pigs reached criterion on a reversal (average of "learners": 276 trials to criterion). None of the pigs learned to discriminate between 2D photos of heads of conspecifics, even after 289 trials, when training was discontinued. Birth weight did not affect learning. We conclude that pigs need input from more modalities than vision alone to enable discrimination between conspecifics.

Abbreviations: LBW = Low-birth-weight, NBW = Normal-birth-weight

Keywords: Pigs – Low-birth-weight – Visual discrimination learning – Facial recognition – 2D portrait photographs – Discrimination task

Introduction

Animals living in social groups must to be able to recognize group members. Recognition of group members is fundamental for establishing and maintaining a stable social relationship and group hierarchy (Sherman et al. 1993). Recognition can take place via different sensory modalities. Most animals use various unique cues and characteristics to build a mental representation of another individual for recognition. Golden hamsters use olfactory cues from different body parts to recognize an individual (Johnston and Bullock. 2001). Ring tailed-lemurs *(Lemur catta)*, for example, are able to use urinary scent marks for discrimination and recognition (Palagi and Dapporto. 2006). A number of species, especially primates such as chimpanzees, rhesus monkeys and lemurs mainly rely on facial discrimination for recognition (Marechal et al. 2010, Palagi and Dapporto. 2006).

Investigations of facial discrimination are no longer restricted to primates, but have been extended to farm animals such as cattle and sheep, which are social animals. Coulon et al. (2009) performed a discrimination experiment with heifers, using 2D photographs of cows. All heifers were able to discriminate between heifers from their own breed (either familiar of unfamiliar) during the training and generalization phase. Almost all heifers could discriminate between cows from different breeds, but needed more trials during the generalization phase. Ferreira et al. (2004) performed a similar experiment with ewes that were first trained with a pair of photos of sheep faces. When they had learned this discrimination, they were shown photos of the same pair of sheep but at an older age in the generalization phase. The ewes learned this transfer more easily than a transfer to a photo pair of totally different individuals. Sheep also seem to learn to discriminate between photographs of conspecifics more easily than between geometric figures (Kendrick et al. 1996).

Pigs also live in groups, so they need to learn to recognise group members individually. A small number of experiments studied social discrimination and recognition in pigs. McLeman et al. (2005) demonstrated that pigs were able to discriminate between other (live) pigs using bimodal sensory cues, or using only one sensory modality. This implies that pigs were able to discriminate between other pigs relying exclusively on visual information. The pigs did not differ in learning ability according to the different sensory modalities.

Ewbank et al. (1974) studied the role of sight in hierarchy formation in pigs. Preventing pigs from seeing each other by putting contact lenses on their eyes did not prevent the formation of a hierarchy. This implies that they could still recognize each other. However, placing ‘hoods’ on the pigs’ faces did prevent hierarchy formation. This could have been caused by the covering of pheromone producing areas.

It is not yet clear whether pigs use visual (facial) cues to discriminate between conspecifics. However, they are able to learn simple visual discrimination tasks. Moustgaard et al. (2004) showed that mini-pigs are able to perform a black-

and-white discrimination task. Contingent on making an error, Moustgaard and colleagues applied 20s of darkness as punishment and a tone as secondary reinforcer. Fourteen out of 16 piglets successfully learned the black-and-white discriminations. Graf (1976) trained 2.5-4 month old piglets on a visual discrimination task with a "Landolt-C" symbol and an "O" symbol. The pigs needed between 120 and 200 trials to reach a level of 80% correct choices. As in the study conducted by Graf (1976), previous studies in our group also showed that pigs cannot be trained quickly to perform a discrimination task using visual stimuli (unpublished results). In contrast, sheep only needed 53 trials to learn to discriminate between a pair of 3-months old unfamiliar lambs, without pre-training with one stimulus (Ferreira et al. 2004). Pigs have a lower visual acuity than humans, sheep or cattle (Entsu et al. 1992, Tanaka et al. 1995, Zonderland et al. 2008). This may explain why pigs need more trials to learn a simple discrimination.

Thus, although pigs appear to be able to learn discrimination tasks with simple shapes as discriminative stimuli, data are inconclusive as to their ability to discriminate between conspecifics, solely based on visual information.

Effects of low-birth-weight on learning

In pigs, due to selective breeding, the number of piglets per litter has increased, and as a consequence the number of low-birth-weight (LBW) piglets has also increased (Quiniou et al. 2002). Research in humans has shown that birth weight and cognitive performance are correlated. Children with LBW often have learning problems and some may suffer from more severe cognitive and emotional issues (Kessenich. 2003). LBW babies of monkeys *(Macaca nemestrina)* have less developed learning abilities in a visual recognition task (Gunderson et al. 1989). Learning problems associated with a LBW are caused by brain injuries in most cases, induced by oxygen deprivation due to insufficient placental oxygen transfer (van den Broek et al. 2010).

LBW piglets may be used as a model for human LBW (Gieling et al. 2012). We recently adopted the spatial holeboard discrimination task for testing pigs. Successful learning in the holeboard task depends on orientation toward distal extra-maze (visual) cues (van der Staay et al. 2012). We found that pigs with a LBW showed delayed learning of the reversal, but not of the original learning task, compared with normal-birth-weight (NBW) siblings. This effect was seen for the working, but not the reference memory component of the holeboard task (Gieling et al. 2012). Lower cognitive abilities in piglets may also have an effect on welfare, due to less control over their environment (Wiepkema and Koolhaas. 1993).

Aim of the study

The aim of the present study was to investigate whether pigs are able to learn a simultaneous discrimination using 2D portrait photographs of conspecifics as discriminative stimuli, and whether birth weight affects their learning.

Because pigs appear to learn visual discrimination tasks slowly, visual discrimination tasks with increasing complexity were presented: 1) a simple discrimination task with one geometric black-and-white stimulus 2) a simultaneous

discrimination task with two geometric black-and-white stimuli, followed by two reversals, and 3) a simultaneous discrimination task with portrait photographs of pairs of pigs as discriminative stimuli. The simple and simultaneous discriminations were included to train the pigs on the procedural requirements of the face recognition discrimination task, and because simple stimuli do not pose a serious challenge for the visual system. The reversal was included to detect possible differences in learning abilities between LBW and NBW piglets, as shown in a study by Gieling et al. (2012). Piglets with a low and a normal-birth-weight were tested. The five best performing animals that had mastered the simple and simultaneous discrimination tasks and their siblings were subsequently trained to discriminate between frontal portrait photographs of pigs. We hypothesized that piglets with a LBW show slight cognitive impairments, and that these may become noticeable during reversal learning. Finally, we hypothesized that because pigs are group-living animals, they need to discriminate and recognize conspecifics. Photographs of conspecifics are biologically more relevant stimuli than geometric symbols, and sheep seem to learn discriminations between conspecifics more easily (Kendrick et al. 1996). Also, mastering the first phase of discrimination learning (phase 1) usually takes the most trials. Therefore we hypothesized that pigs are able to discriminate between photographs of conspecifics and that they would learn the photograph discriminations in fewer trials than the acquisition of the simple discrimination in phase 1 of the experiment.

Material and methods

Ethics note

The study was reviewed and approved by the local ethics committee (DEC, dierexperimenten¬commissie), and was conducted in accordance with the recommendations of the EU directive 2010/63/EU. All efforts were made to minimize the number of animals used and to avoid suffering.

Subjects

Eighteen female piglets (nine sister pairs), born at a commercial pig-breeding farm (cross-breds Duroc X Yorkshire and Duroc X Danish landrace) were used in the experiments: nine animals with a LBW and nine with a NBW. Only females were used, because males were routinely castrated to prevent development of boar taint later in life.

In order to determine which piglets were born with LBW, all newborn piglets were weighed on day three after birth (day of birth = 0). Then, the overall mean and overall standard deviation of these litters plus all other litters weighed in the course of one year (40 litters in total) were defined. The calculated overall mean and standard deviation were used to define LBW: LBW is a weight that is smaller than the overall mean minus at least 1x the standard deviation.

From each litter, the piglet with the lowest birth weight that fulfilled the criterion of LBW, behaved lively and looked vital was chosen. To define NBW, a new mean weight *per litter* was calculated after excluding all LBW piglets. The female piglet with a birth weight closest to this new mean was chosen from every litter and served as normal weight control.

Piglets' tails were docked at the age of three days. The piglets were weaned from the sow at 3 ½ weeks of age. One pig died two weeks after starting the habituation period because of acute pneumonia. Consequently, the study was continued with 17 piglets.

Housing conditions

After weaning the piglets were group-housed in two adjacent former horseboxes, adapted for housing piglets. The stable was naturally ventilated and lighted. The pens measured 4.95 x 4.0 meters and contained a piglet 'nest'. The floors were covered with straw and some toys were provided. A radio was playing between 7:00-19:00. Siblings stayed together and each pair of sibling was randomly assigned to one of the two pens. Therefore, one pen consisted of seven piglets (as one piglet died) and the other of ten piglets. Ambient day temperatures, which were similar inside and outside the building ranged from about 10° Celsius in October to about 35° Celsius in July.

The animals received about ⅓ of their total amount of food per day in the morning, and ⅔ in the afternoon (scatter feeding). The piglets received normal quantities of commercial piglet food during their growth phase. Water was provided *ad libitum* in each pen via an automatic drinking nipple. Two months after the start of the study, the pigs switched to food for breeding sows that is lower in energy, in order to slow down their weight gain.

All piglets were weighed on day 3 after birth, and at the ages of approximately 12, 15, 23 and 32 weeks.

Discrimination task: apparatus

The apparatus in which the visual discriminations tasks were conducted is depicted in Fig. 1B. The light conditions in the setup were relatively stable. Skylights present in the testing area were covered to avoid variable illumination of the arena by sunlight. The test environment was illuminated by a bright gas discharge lamp (Agrilight AL2007, 250W, Monster, the Netherlands). On animal level approximately 640 lux was provided.

Visual stimuli

Visual stimuli A and B (see Fig. 2) measured 175 x 175 mm and were printed on white paper. Comparable symbols but smaller and printed on A4 paper had also successfully been used in an unpublished pilot simultaneous discrimination study. In the present study, a black symbol on a white background and a white symbol on a black background were chosen to facilitate discrimination. Visual stimuli C–G (Fig. 2) were 277.5 x 277.5 mm and were taken about four weeks before the experiment started. These stimuli representing images of conspecifics covered an area approximately twice as large as the simple symbols (stimuli A and B) to make details better visible, thus facilitating detection of differences between the photographs of the pig heads. As we do not know how pigs (visually) discriminate between conspecifics, we choose pairs that humans could easily discriminate. Care was taken to choose photo pairs of pigs that differ in various aspects such as color, position of the ears, spots or no spots

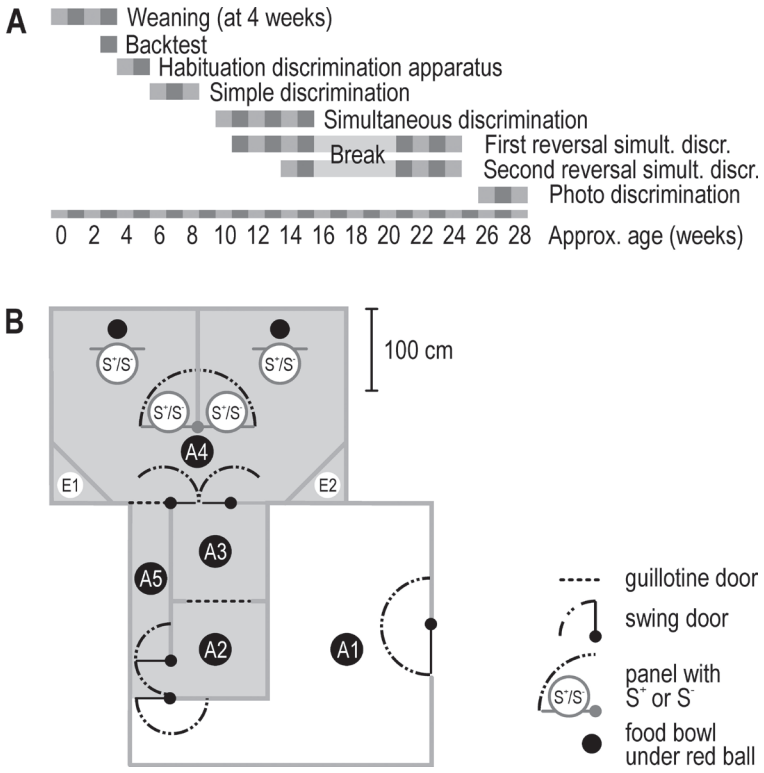
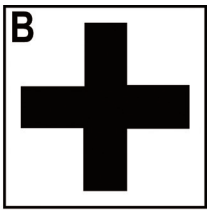
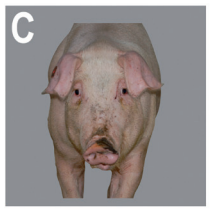
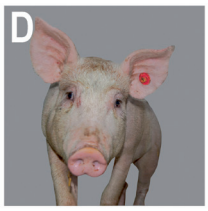
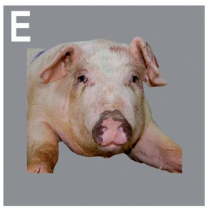


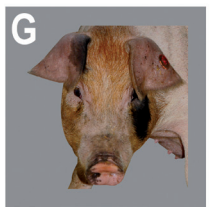
Fig. 1. Panel A: timeline of the study: not all animals learn at the same speed, therefore some overlap occurs between tests over time. **Panel B:** floor plan of the test apparatus and adjacent waiting area. During simultaneous discrimination learning, the pigs were transferred to the next phase after reaching the respective criterion, except for the photo discrimination task which was started when all pigs were approximately 26 weeks old. Note that there was a break of 5 weeks in which no training was given (due to construction work in the stable). During training sessions, a pig stayed in the waiting area (A1), together with all other pen mates, until it was tested. At the start of daily training session, a pig entered A2 of the testing apparatus and stayed there while the experimenters prepared the visual stimuli. Doors could be opened from the outside with a system of ropes and pulleys. Then, the pig entered A3, where it could see the visual stimuli through a double swing door. After about one second, this door was unlocked and the pig pushed it open with its snout and walked left or right in A4 (240 X 360 cm). Two pairs of frames depicted the visual stimuli (S^{plus}/S^{minus}) and signaled where the bait could be found. The reward was placed in a food bowl under one of the red balls in A4. The pig had to lift the ball that covered the food trough with its snout to find the reward. Three inaccessible M&M's were placed underneath a false bottom in both food bowls, to prevent olfactory bias. After a pig had chosen –touching and/or lifting one of the balls and consuming the reward, if the correct side was chosen –it left the apparatus via corridor A5 and returned to A2, or to the waiting area (A1) after completion of all training trials. Care was taken that the pig did not cross to the other side of the apparatus after choosing. The two experimenters who stood behind partitions E1 and E2 during testing guided the pig to A5. Eye contact with the animals was avoided.











H

Pig	S^{plus}	S^{minus}
5	D (pig 7)	F (pig 9)
6	D (pig 7)	F (pig 9)
8	C (pig 5)	G (pig 10)
7	C (pig 5)	G (pig 10)
10	E (pig 8)	C (pig 5)
9	E (pig 8)	C (pig 5)
12	E (pig 8)	F (pig 9)
11	E (pig 8)	F (pig 9)
17	D (pig 7)	G (pig 10)
15*	D (pig 7)	G (pig 10)

Fig. 2. The geometric visual stimuli depicted in panels A and B were used to train pigs in the simple discrimination task. For pen 1, stimulus A was the Splus. For pen 2, stimulus B was S^{plus}. The portrait photographs of pigs depicted in panels C-G were used as stimuli in the photograph discrimination task. The 5 pigs performing best in the simple discrimination task and their sibling sisters* were used in the photograph discrimination task (e.g.: pig no. 10 was one of the 5 best performing pigs, and pig no. 9 is her sister). Pigs used came from pen 1 and pen 2. Which photographs were used as S^{plus} and S^{minus} is listed in panel H (C–G in panel H refer to the corresponding photographs).

* Note: instead of testing pig no. 18, the sister of pig no. 17, we decided to test pig no. 15, because pig no. 18 behaved highly unmotivated during previous testing.

etc. The photos were manipulated in Adobe Photoshop: the original background of the images was removed and replaced by a neutral gray background. The proportions of the original pictures were altered so that the ratio between image and background was similar in all photographs. All photographs showed pigs from the present study.

The photos used in the photo discrimination task (Fig. 2C−G) were larger than the simple geometric visual stimuli (Fig. 2A, B). Therefore, the frames in which the photos were placed were also larger (65 x 65 cm). The frames at the front were made mobile; they could swing by means of a hinge if a pig pushed against them

(see Fig. 1B). This was done in order to allow the pigs, which had grown to a weight of 85 kg on average when starting training on the photo discrimination task, more space to move in the apparatus.

Procedures

During testing, a radio was playing to mask sudden unexpected background noises.

Habituation

Before the training started, a stepwise shaping procedure was conducted: four days after the piglets were housed in their new pens, habituation started for a period of twelve consecutive working days. Animals were first habituated to the experimenters, the corridor and waiting area during two daily 30-minute sessions. First, all animals of a pen entered the test apparatus, then in groups of 3-4, then in pairs, and finally they entered the apparatus individually (two sessions a day for 45 minutes). The piglets were introduced to the flashlight used in the experiment and learned to lift the red balls with their snout to get bait consisting of 1 M&M chocolate (see Fig. 1). 3 M&Ms were put under both the left and right ball, to prevent the development of a side bias or alternation pattern. No visual stimuli were presented during the habituation phase.

Simple discrimination: acquisition

The piglets entered the testing apparatus individually, in a fixed order. Only one visual stimulus was used (see Fig. 2A,B), which was shown randomly left or right. The piglets from the two different pens were assigned a different rewarded stimulus (see Fig. 2). When the piglet stood with its head faced towards the folding doors of the apparatus, a flashlight was flashed three times on the stimulus in order to draw the pig's attention towards the stimulus.

During the first 14 testing sessions, training the pigs was very time consuming. Therefore, the sequence of testing the two groups changed every day: 1-2-1, 2-1-2, 1-2-2, 2-1-1 and so on (where 1 and 2 refers to pen 1 and 2), i.e., one of the two pens received one session (10 trials) a day and the other pen two sessions a day. After 14 sessions, the piglets performed fast enough to run two daily sessions with both pens. A choice was defined as the animal touching a ball with its nose. The animals could thus approach the wrong position without touching the ball, then turn back and choose the correct side. A correct choice was rewarded with one M&M chocolate, found when lifting the ball.

During the first 50 trials the piglets were allowed to go to the correct stimulus side after making a mistake and eat the M&M (in order to learn that there is always a stimulus that is rewarded). At the beginning of testing, some animals tried to leave the testing area after some trials and were allowed to go out. Their session was continued (and almost always completed) after the other pigs had completed their daily training session(s). One piglet showed a strong side bias (left) at the start of the experiment and therefore the stimulus was placed at the opposite (right) side until no side bias was observed anymore. To prevent lack of motivation to perform, a forced trial was given after making mistakes leading to no reward in more than 4 consecutive

trials: in this trial the animal could only reach the correct stimulus and therefore received a reward.

Primarily, we had defined a learning criterion of eight correct trials in a series of ten trials during two successive sessions (80%). However, in the course of testing we observed that some piglets were able to reach this criterion by responding inflexibly across consecutive trials, for example by alternating continuously. This behaviour increased the probability to score 80% correct trials by chance. In particular if a pig persistently alternated, and if the scheduled presentation was roughly left-right-left-right, and the pig made the correct choice during the first trial, they could reach this criterion easily without further evidence of paying attention to the position of the S^{plus}. Therefore we changed the criterion to a score of 80% correct choices in each of two successive blocks of 10 consecutive trials. Animals were trained until reaching this new criterion. Consequently, at a certain moment, one pig could be still in the simple discrimination phase, while another one had already reached the following phase(s) of the experiment.

Simultaneous discrimination of black-and-white figures: acquisition

Two stimuli, a rewarded S^{plus} and an unrewarded Sminus (see Fig. 2A,B), were shown simultaneously at the left and right side. Their position was determined by random schedule. The stimulus rewarded during the simple discrimination task was still rewarded, but a flashlight was no longer used to draw the pig's attention to the rewarded stimulus. The same criterion for learning as used in the simple discrimination task was applied.

Simultaneous discrimination of black-and-white figures: first reversal

The previously rewarded stimulus was no longer rewarded. Instead, the previously unrewarded stimulus now became rewarded.

Simultaneous discrimination of black-and-white figures: second reversal

During the last phase, the first S^{plus} that had been rewarded during acquisition of the simple and simultaneous discrimination, and that was unrewarded (S^{minus}) in the first reversal, was rewarded again.

End of training

For practical reasons training was discontinued when all pigs being trained in the first reversal had received at least the number of trials that was the median number of trials (100) to reach criterion in the acquisition phase of the simultaneous discrimination. Consequently, the phase in which a pigs' training was discontinued differed between piglets.

Simultaneous discrimination of portrait photographs of pigs: acquisition

For the photo discrimination task, the five best performing gilts from the previous discrimination task and their siblings were selected. Rewards were doubled (2 instead of 1 M&M) because of the pigs' weight gain.

Only ten pigs instead of all seventeen were used, in order to have more time per pig and therefore to be able to run more trials per day. At the start of the photo experiment, the animals were about six months old. Before starting training on this task, the pigs received a short habituation session of six trials to get used to the new photo frames. No photos were shown and M&M rewards were available at both sides.

Pairs of photographs were used, which were interchanged semi-randomly left or right (max. 3 consecutive times on one side). Siblings were assigned the same pair of photographs (see Fig. 2). From these pairs one photo was selected to serve as S^{plus}. Every correct choice was rewarded with two M&M's. Each session consisted of ten trials. The pigs received at least one session a day and a maximum of three sessions a day.

Training was discontinued after reaching criterion or 289 trials, whichever event occurred first. The same criterion was utilized as in the simple discrimination task: a score of four times 80% correct in a series of five sessions. The maximum of 289 trials was based on the median of the number of trials till criterion found in the simple discrimination task.

Based on the median of the number of trials till criterion in the simple visual discrimination task, we decided to discontinue training of the pigs after they had reached the criterion, or when they had received a maximum of 289 trials on the simultaneous discrimination of photographs, whichever event occurred first. We established this criterion because we argued that if pigs would use face recognition in their daily life, they would learn this task relatively easily, as the stimuli would have intrinsic relevance. Further, they had already shown that they understood the principles of the discrimination task with the geometric stimuli.

Statistics

All data are reported as means and standard errors of the mean (SEM).

Birth weight and weight gain: differences in birth weight and weights at 12, 15, 23 and 32 weeks of age were analyzed by an analysis of variance (ANOVA) with the repeated measures factor Age in weeks. Number of trials to reach criterion in the discrimination tasks was analyzed with a one-factorial ANOVA with the factor Birth weight (LBW vs. NBW).

Simple discrimination learning: in order to analyze whether pigs started discrimination learning with a preferred strategy (e.g. successive alternations, strong side bias) we calculated the number of correct choices and alternations per session for the first 15 sessions (3 blocks of 5 sessions), according to the method described in detail in (Spowart-Manning and van der Staay. 2004). We chose for

the first 15 sessions because after 15 sessions, the first pig had learned the simple discrimination task. For example, the rewards were available in the sequence R R L R L R L L R L in a block of 10 successive trials. A pig chose **R R L R L** L *R R **R** R*. The six correct choices are printed bold, and the longest series of choices to one side (here right) is printed in italics. All these variables were expressed as percentages of number of trials run in a session (normally ten, but incidentally less, for example due to lack of motivation of a pig to perform the task).

Effects of birth weight on the preferred strategy (longest series and alternations) were analyzed by ANOVA with the between subjects factor Birth weight and the within subjects (repeated measures) factor Blocks. Effects of birth weight on the number of trials to reach criterion in the simple and simultaneous discrimination task and the reversals of the simultaneous discrimination task, were analyzed by ANOVA with the factor Birth weight.

Results

Birth weight and growth

The mean birth weight of the LBW group was lower than that of the NBW group ($F_{1,15}$ = 35.66, p<0.0001, Fig. 3A). Repeated measures analysis revealed that on average, the body weight of the LBW group was lower than that of the NBW group over the period from birth to the age of 32 weeks ($F_{1,15}$ = 12.28, p = 0.0032). The LBW piglets gained weight slower than the NBW piglets (Age in weeks, $F_{4,60}$ = 975.41, p<0.0001; Birth weight by Age in weeks interaction, $F_{4,60}$ = 8.84, p<0.0001), i.e. the weight differences between the two groups were persistent (Fig. 3B).

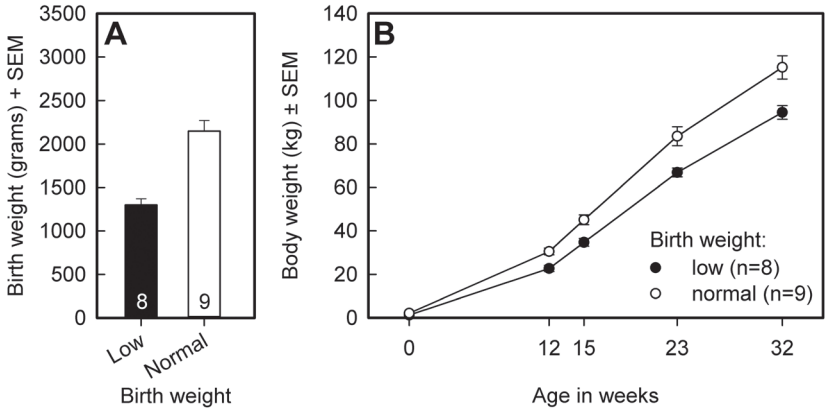


Fig. 3. Birth weight (grams; panel A) and growth (kg; panel B) of piglets born with a low or normal-birth-weight. Means, standard errors of the means (SEM), and number of animals per group are shown.

Simple discrimination

Choice strategy during the first 3 blocks of 5 sessions of the simple discrimination task

Percentage correct choices: averaged over the first 15 acquisition sessions

(3 blocks of 5 sessions each) of the simple discrimination task, no differences were found between the low and NBW for the percentage correct choices. The percentage correct choices decreased over the three blocks (Blocks: $F_{2,30} = 20.42$, $p < 0.0001$) similarly in both groups (Birth weight by Blocks of sessions interaction; $F_{2,30} = 0.13$, $p = 0.882$; see Fig. 4A).

Percent alternations: The two groups did not differ in the average percentage alternations during the first 15 sessions (Birth weight: $F_{1,15} = 0.62$, $p = 0.444$). Across the three successive blocks, the percentage alterations increased (Blocks: $F_{2,30} = 68.44$, $p < 0001$) similarly in both groups (Birth weight by Blocks of sessions interaction: $F_{2,30} = 1.38$, $p = 0.266$; See Fig. 4B).

Percent longest series: The LBW and NBW piglets did not differ for the percentage longest series (Birth weight: $F_{1,15} = 0.07$, $p = 0.789$) in the first fifteen sessions. However, both groups showed a decrease in the percentage longest series over the three successive blocks of five sessions (Blocks: $F_{2,30} = 28.12$, $p<0001$). This decrease was similar for the two groups (Birth weight by Blocks interaction of sessions: $F_{2,30} = 0.03$, $p = 0.975$; see Fig. 4C).

Latency to choose: the average latency [log (s + 1)] to choose a food bowl (i.e. touching the ball) of piglets in the LBW group was shorter than that of the pigs in the normal weight group ($F_{1,15} = 5.87$, $p = 0.029$; see Fig. 4D). Trials to criterion, acquisition of the simple discrimination task: the two weight groups did not differ in the number of trials needed to reach criterion ($F_{1,15} = 2.04$, $p = 0.173$; see Fig. 4E). All piglets reached the criterion of learning of the simple discrimination task. LBW piglets needed on average 386 trials and NBW piglets 294 trials to reach criterion.

Trials to criterion, acquisition of the simultaneous discrimination: fifteen of the 17 piglets reached criterion in the simultaneous discrimination task. Of the piglets that did not learn the task, one piglet was from the LBW group and one from the NBW group. The groups did not differ for the number of trials needed to learn the task ($F_{1,13} = 0.10$, $p = 0.753$; see Fig. 4F). LBW piglets needed on average 93 trials to master this phase and NBW piglets 103 trials.

Trials to criterion, first reversal: only five of 15 piglets reached criterion performance in the first reversal. The number of animals reaching criterion was not different between groups (2 of the 7 LBW pigs and 3 of the 8 NBW pigs reached criterion; Fisher Exact probability, $p = 1$). Regarding only the data of the animals reaching criterion, no differences were found for the number of trials to reach criterion between the two groups (LBW, 237 ± 57 trials, $N = 2$; NBW, 302 ± 44 trials, $N = 3$; $F_{1,3} = 0.84$, $p = 0.427$). LBW piglets reached criterion with an average of 289 trials and NBW piglets in 345 trials.

Trials to criterion, second reversal: none of the piglets reached criterion during the second reversal of the simultaneous discrimination task. The five pigs received between 60 and 350 trials during the second reversal. Training was stopped because

the pigs that were in the first reversal phase had received minimal the median of the number of trials in the acquisition of the simultaneous discrimination.

Photo discrimination

We set a criterion of a maximum of 289 trials (which was the median of the number of trials needed to master the simple discrimination task). Training was discontinued after all animals had received 289 trials. At that point, none of the pigs had reached criterion of discriminating between the photos.

Discussion

All 17 pigs reached criterion in the simple discrimination task, whereas 15 pigs reached criterion in the simultaneous discrimination task with two geometric figures. Only five pigs were able to reach criterion in the first reversal (on average 289 trials for LBW pigs and 345 for NBW pigs), and not one reached criterion in the second reversal of the simultaneous discrimination task. The five pigs received respectively 60, 100, 250, 300 and 350 trials in the second reversal. The five piglets that performed best in the simple discriminations task and their sisters (with one exception, see Fig. 1H) were trained on the photo discrimination task. None of these pigs was able to discriminate between faces of conspecifics using 2D photographs within the predetermined maximum number of 289 training trials. Considering the biological relevance of being able to discriminate between conspecifics (Zayan and Vauclair. 1998), we expected that the pigs would learn this discrimination quickly, provided they are able to discriminate between individuals if exclusively offered visual information. The results contrast with studies done with sheep and cattle (Coulon et al. 2009, Ferreira et al. 2004). We cannot, however, exclude that the pigs eventually would have learned the discrimination if training was continued, but these results indicate that pigs probably do not use facial discrimination in daily life.

The difference in body weight between the LBW and NBW groups persisted until the end of the study, when the animals were approximately 28 weeks old, corroborating earlier findings e.g. (Powell and Aberle. 1980, Rehfeldt and Kuhn. 2006) and our own previous (unpublished) observations. Birth weight had no effect on learning of any of the discrimination tasks. These results contrast with the finding by Gieling et al. (2012) that low-birth-weight retarded acquisition of a reversal in a spatial holeboard task. The measures in the visual discrimination task are probably less sensitive and measure less diverse cognitive domains than the spatial holeboard task. The large number of trials necessary to learn the simple discrimination task suggests that the task was very difficult for all pigs. A very high task difficulty reduces the chance to detect (subtle) differences between groups. Motivational issues could have played a role in the slow learning process; no negative consequence followed after a making wrong decision and by chance within a ten trial session a maximum of 10 M&M's could be found. This average reward percentage could have been high enough for most pigs to not substantially try to improve their performance by learning the task rules.

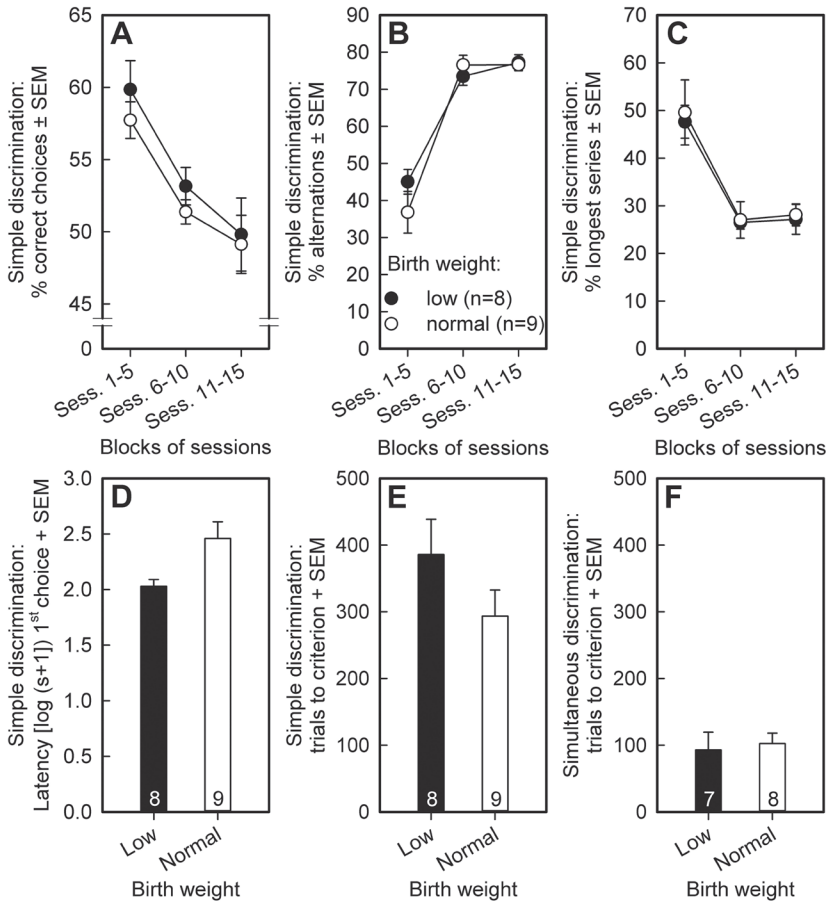


Fig. 4. Percent correct choices **(panel A)**, percent alternations **(panel B)** and longest series of successive choices to the same side [left (L) or right (R), **(panel C)**], and latency to first choice **(panel D)** during the first three blocks of five training sessions on the simple visual discrimination task, and trials to criterion on the simple **(panel E)**, and simultaneous discrimination task **(panel F)** are shown as means and standard errors of the mean (SEM).

Simple discrimination

Analysis of the choice behaviour during the first 15 sessions of the simple discrimination task showed that pigs quickly developed alternation behaviour that increased across sessions, whereas the length of series of longest choices to one particular side decreased, along with a slight (approx. 10%) decrease of correct choices. It may be possible that this decrease is due to less motivation or to the increased alternation behaviour. Overall, the piglets needed a large number of trials to master the different discrimination tasks. Most piglets needed hundreds of trials to acquire the simple discrimination task (170-615 trials). This is even more than the 120-200 trials found by Graf (1976) and Gieling et al. (2012). It is possible that learning went slowly because pigs have low visual acuity. Pigs could not discriminate visual cues below 20 mm in an experiment by Zonderland et al.

(2008), thus their visual acuity is lower compared to sheep (Tanaka et al. 1995). However, learning the simultaneous discrimination task went much faster than the simple discrimination (within approx. 100 trials). The reversal of the simultaneous discrimination tasks was learned very slowly, and only 5 of 15 piglets reached criterion. This result is in line with the observation of Moustgaard et al. (2004) that learning the reversal in a visual discrimination task in Göttingen minipigs was slower than the original learning.

In the present experiment, all pigs were trained with massed trials, i.e. multiple trials were run in close succession. It has been shown that spaced trials are more effective than massed trials to acquire learning tasks (e.g. Klapdor and van der Staay. (1998) and Sisti et al. (2007)). Moreover, spacing of trials may reduce spontaneous alternation, because the chance increases with increasing inter-trial interval that an animal might forget which side it chose in the previous trial. Spontaneous alternation may explain partly why the animals did not learn fast; when alternating most of the trials, they receive on average about five rewards (M&Ms) per session, i.e. approximately 50% of all trials are rewarded. This reward density may suffice to maintain the alternation behaviour. Many species show predominantly win-shift behaviour in situations where food sources are depleted (van der Staay et al. 2012). Pigs have also been observed to use a win-shift strategy in a study by Laughlin and Mendl. (2000). Consequently, the animals may not easily detect the contingency underlying the task, namely that one stimulus predicts reinforcement. Applying spaced trials, though, would imply that the study would be extremely more time consuming.

Applying punishment may also speed up learning. Moustgaard et al. (2004) punished pigs with 20s of darkness, contingent on incorrect choices in a black-and-white discrimination task. In addition, they used a tone as a secondary reinforcer, contingent on correct choices. Under these experimental conditions, fourteen out of 16 Göttingen minipigs learned the visual discrimination, needing a median number of 7 sessions with 20 trials each to reach criterion. Direct comparison between our study and the other study Moustgaard et al. (2004) is not possible, because of numerous procedural differences. It remains therefore to be investigated whether applying punishment and/or introduction of a secondary reinforcer speeds up learning of visual discriminations in pigs.

Discrimination of portrait photographs of pigs

The pigs failed to acquire the discrimination between portrait photographs of conspecifics in 289 trials. In contrast, sheep needed only 53 trials to learn to discriminate between a pair of 3-months old unfamiliar lambs, without pre-training with one stimulus (Ferreira et al. 2004). The result of the present study is also in contrast with facial photo discrimination experiments in cows, which needed on average 40 trials to learn a discrimination (Coulon et al. 2009, Ferreira et al. 2004). Pigs and sheep also differ in that for sheep it is easier to discriminate between faces of conspecifics than between geometric stimuli (Kendrick et al. 1996). Our pigs learned the simple discrimination task with a median of 289 trials, but in the same number of trials none of the pigs learned the photo discrimination task. However, for sheep faces are ‘special’: sheep have neural circuits in the temporal cortex

that respond selectively to faces. These face-selective responses are influenced by different factors, such as species, breed and familiarity. Some cells discharge more in the presence of frontal views of sheep whereas other cells have a preference for human and dog faces (Kendrick et al. 1996). Whether similar specialized neuronal circuits exist in pig has not yet been investigated.

So why did the pigs fail in mastering this discrimination task? Pigs are likely to rely more on olfactory and auditory cues in their daily life than on visual cues (Jensen 2002). This was confirmed by a study of Hutson et al. (1993). In that study, sows responded more to auditory and olfactory stimuli (playback of sow grunt, eucalyptus oil) than to visual stimuli (novel rod). However, Croney et al. (2003) reported that pigs learned to use visual cues to locate a food source, although learning was slightly better when provided with olfactory cues. Contrary to rodents, well validated learning and memory tasks suited for testing pigs are still scarce (Gieling et al. 2011), and the role of the different sensory modalities in learning still needs to be elucidated. In conclusion, there is only weak scientific information about the way pigs use vision in daily life.

It may be possible that pigs can discriminate between live conspecifics using facial cues, but just not between photographs of conspecifics. Ryan and Lea. (1994) found that pigeons could discriminate between live conspecifics in a behavioural habituation experiment, but did not learn a discrimination task with pigeon photos and did not react to video images of pigeons. Bovet and Vauclair (2000) point out that "(…) pictures, being still or in motion, are abstractions from the reality they represent. Thus, even if animals predominantly use visual cues to identify social stimuli, auditory and olfactory information are also present in encounters with real conspecifics but absent in pictorial". Moreover, pictures are abstract stimuli that lack the information of perceiving depth, and colors and size can be different from the 'real world' (Bovet and Vauclair. 2000). We therefore tentatively conclude from the results of our study that pigs are unable to discriminate between portraits of conspecifics presented as 2D photographs. Another possibility is that pigs do see photographs as representations of conspecifics, but that they are just not able to discriminate conspecifics visually.

From the results of this experiment, we cannot conclude whether pigs see pictures as representations of other pigs or not, although we have some indications (unsystematic behavioural observations) that they do: they reacted differently to the photo stimuli than to the geometric stimuli. When the pigs noticed the photos for the first time they all stood tense and motionless, appeared to stare at the photos. Some pigs even jumped backwards and squealed briefly in response to presentation of the photos. Their reaction was clearly distinct from the untouched behaviour in geometric stimuli at the start of the simple and simultaneous discrimination tasks.

It would be interesting to further investigate whether pigs do show visual recognition if confronted with live animals. To answer this question, it would be necessary to perform an experiment in which factors other than vision are

excluded. McLeman et al. (2008) tried to control for information via other sensory input channels by ventilating air from an animal-free room. Vocalizations were not masked, but a sound system was used to replay the sounds of the two stimulus pigs at four different locations around the test apparatus. In this experiment, two out of three piglets appeared to be able to discriminate between pigs using only visual information. Therefore, it might be possible that pigs discriminate better (pay more attention to stimuli) when using live, mobile stimuli or that they need whole bodies and behaviour in order to discriminate visually. However, in the experiment (McLeman et al. 2008), the sample size was very small and there was no hard evidence that the pigs could not use non-visual information such as odour cues. Kristensen et al. (2001) also points out that the visible presence of other pigs provided stronger stimulation of approach in their experiment with a Y-maze and that information from various sensory systems seems to facilitate recognition.

Effect of birth weight

Birth weight does not influence visual discrimination learning of geometric patterns in pigs; no differences were found between the groups for the number of trials needed to reach criterion of learning in the simple and simultaneous discrimination task and its reversal. These results contrast with some reports about the effects of low-birth-weight on learning in humans (Kessenich. 2003) and monkeys (Gunderson et al. 1989). In line with our results, Westwood et al. (1983) did not find learning impairments in term born LBW children. Piglets and human neonates suffering from LBW are treated differently after birth. These piglets are less likely to survive than human babies provided with medical care. As a consequence, the group of LBW piglets is biased in that it represents survivors, because we could only choose from piglets that survived without extensive medical attention. In the study (Gieling et al. 2012) with the cognitive holeboard, differences between LBW and NBW piglets were found in learning the reversal. In the present study, only five piglets had mastered the reversal, with no difference between the two birth weight groups. We tentatively conclude that LBW does not influence learning ability in pigs in visual discrimination tasks. However, in general visual discrimination tasks apparently are not very suitable for detecting learning differences in piglets, because they need a very large number of trials to learn the different contingencies and after a period of training motivation seems to decrease.

Piglets with a LBW needed less time from opening of the folding doors until touching the ball, i.e. chose faster than the NBW piglets. This could indicate that they had a stronger motivation to gain a reward. However, we observed that the bigger pigs in general moved slower and that may also explain the difference (supported by positive correlations between latency to choose and weights at 12, 15, 23 and 32 weeks of age in that order: 0.50, 0.55, 0.66 and 0.63, all with $p < 0.05$). No differences in the speed of choosing were observed in a spatial holeboard discrimination task between LBW and NBW pigs (Gieling et al. 2012).

Recommendations

For future studies assessing discrimination between conspecifics, spaced trials instead of massed trials may be used to prevent alternations (which is an indication of good spatial working memory, but may interfere with learning to discriminate between S^{plus} and S^{minus}) and to speed up acquisition of the task. However, this would imply that fewer pigs can be trained with the same number of daily trials, or that fewer trials per day can be run with the same number of animals.

As outlined in the Animals, materials and methods section, we needed to redefine criteria during the course of the running experiment. The first criterion of 80% corrects responses in two consecutive sessions seemed to be less suitable: we got the impression that some piglets had met this criterion without responding to the contingencies of this task. This may be prevented by using a pseudorandom schedule instead of a random schedule. The pigs showed strong alternation behaviour. We suggest adopting a stricter criterion of 4 sessions with 80 percent correct responses during 5 consecutive sessions from the beginning in future experiments.

For studying the effects of LBW on pig cognition, another test may be used such as the cognitive holeboard, which pigs learn more easily and is probably more sensitive (Gieling et al. 2012). For studying the discrimination of conspecifics, stimuli that provide information (such as emitted by living pigs) via additional sensory modalities may be used.

Conclusion

In conclusion, the results from the photo experiment show that pigs are not able to discriminate between conspecifics by means of 2D portrait photographs.

The results of this study further show that birth weight does not affect learning ability in a visual discrimination task in pigs but that it is correlated to motivation.

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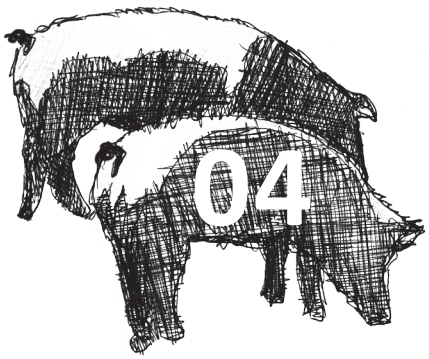

Cognitive performance of low- and normal-birth-weight piglets in a spatial holeboard discrimination task

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Abstract

Introduction: Learning impairments are often seen in children born with low-birth-weight (LBW). A model with translational value for long-term effects of LBW in humans is needed to further our understanding of how LBW and cognition are related. The similarities between development stages in human infants and piglets, and the high prevalence of LBW piglets make them a naturally occurring potential model in which to study cognitive impairment associated with LBW in humans.

Results: Although both groups learned the configurations and rapidly reduced the number of incorrect visits, the LBW piglets showed a transiently retarded acquisition of the first reversal.

Discussion: The results of the experiment support the hypothesis that LBW is related to (mild) subsequent cognitive impairments. In the future, piglets may be suitable models for testing the effects of putative therapeutics.

Methods: To examine this potential model, we tested pairs of LBW and NBW (normal-birth-weight) piglets in a spatial holeboard (a matrix with 4 × 4 holes in the floor) task during one acquisition and two reversal phases in their own individual configurations of rewarded holes.

Introduction

In humans, being born with a low-birth-weight (LBW) after being carried to term (small for gestational age (SGA)) is a phenomenon with a fairly high prevalence that has been extensively studied. LBW occurs in the United States in 8% (2002–2003) of all neonates despite advanced prenatal care (Ergaz et al. 2005). In some developing countries 16–50% of term infants are SGA, and the poor home environment in many of the countries adds to the risk of suboptimal development in these children (Gardner et al. 2003). Various studies in humans have shown that being born underweight is a risk factor for cognitive deficits (Chaudhari et al. 2004, Frisk et al. 2002, Ido et al. 1995, Kessenich. 2003, Martinussen et al. 2009, Silva et al. 2006), learning problems (Frisk et al. 2002, O'Keeffe et al. 2003), spatial orientation difficulties (Leitner et al. 2005), attention problems (O'Keeffe et al. 2003), depression (Raikkonen et al. 2008), reduced brain volume (Martinussen et al. 2009), and reduced academic achievement and professional attainment (Strauss. 2000) later in life.

In commercial pig rearing, selection for high fecundity in the sows and consequent increases in litter size have resulted in more piglets being born with LBW in each litter (Beaulieu et al. 2010, Milligan et al. 2002, Quiniou et al. 2002). LBW is associated with a higher risk of pre- and postnatal mortality and preweaning deaths (Gondret et al. 2005, Milligan et al. 2002, O'Reilly et al. 2006). However, a considerable number of vulnerable underweight piglets survive the critical period after birth.

Growth retardation in the pig fetus is suggested to be an adaptive response to poor perfusion to maintain pregnancy (Blomberg et al. 2010). The meat production consequences of being born underweight have been studied extensively (Gondret et al. 2005, Quiniou et al. 2002, van der Lende and de Jager. 1991). However, nothing is known about the effects of being born underweight on the cognitive capacities of these piglets if they survive. Being born with LBW may have implications for animal welfare if, for example, the ability to adapt to the housing and management conditions is impaired because of insufficient ability to control the environment and reduced adaptive capacities (Broom. 1991).

LBW piglets, like undersized human neonates, show characteristics of immaturity/dismaturity. They are likely to develop postnatal complications (Cooper. 1975). We hypothesized that the underweight piglet may be a useful biomedical model for studying implications of LBW in humans, and for assessing, in future research, the effects of putative therapeutic interventions that ameliorate the adverse consequences of SGA.

It has been reported that the hippocampus is a significant predictor of cognitive functioning in human adolescents who were born SGA (Martinussen et al. 2009). The cognitive holeboard task is a hippocampus dependent task (Oades and Isaacson. 1978). Therefore, we tested the hypothesis that birth weight influences cognitive performance of pigs. We did this by using a holeboard task that is suited for assessing spatial learning and memory in pigs (Arts et al. 2009) and enables the measurement of several behavioral domains (Gieling et al. 2011).

Methods

Ethics Note

The study was approved by the ethics committee at Utrecht University, and was conducted in accordance with the recommendations of the EU directive 86/609/EEC. All efforts were taken to minimize the number of animals used, and also to minimize their suffering.

Animals

Pigs ((Terra × Finnish landrace) × Duroc) were bred at the farm at Utrecht University under conventional Dutch commercial pig housing conditions. Nine pairs of female piglets were selected (each pair from a different litter), based on the body mass measured on day 3 after birth (= day 0). Male piglets were not selected, because they were routinely castrated.

Each pair consisted of one LBW piglet and one NBW piglet. Based on the average weight and SD of a litter of piglets, all those with a body mass at least 1 SD below the litter average were defined as LBW. Of these, the female piglet with the lowest body weight was selected. Next, the average weight of the litter was recalculated excluding all LBW piglets, and the female with a weight closest to this newly defined litter average was selected as the NBW piglet.

Housing

The piglets were mixed and moved to the experimental unit 2 days after weaning, at 4 weeks of age. Litter pairs were randomly assigned to one of two adjacent, enriched pens, each measuring 4 × 5 m, in a naturally ventilated stable. Consequently, one pen housed four pairs of piglets, and the other housed five pairs. Minimal and maximal temperatures in the stable were registered daily, and ranged between 3 °C and 31 °C.

Each pen contained a covered piglet nest, and straw bedding covered the concrete floor. The ambient temperature and humidity conditions inside the nest fell within the piglets' thermoneutral zone. Food and water were provided *ad libitum* to all animals until week 10 of the experiment. To see whether motivational differences were associated with changes in feeding schedules, we introduced the following feeding schedule during the final 4 weeks of the experiment: 10 of the animals (which were housed together) were fed ⅓ of the daily food amount ~1 h before the start of behavioral testing (~8 am) and the remaining ⅔ of the daily food was fed after testing, at ~4 pm; the other eight animals were fed *ad libitum*. The building had natural lighting and a radio played between 7 am and 7 pm.

Testing Room

The testing room was located adjacent to the housing room, and the animals could access it through a corridor leading to a (straw-enriched) waiting area. The testing apparatus contained an abundance of extra maze cues. All animals housed together in a pen were let out through the corridor to the waiting area at the same time, and were tested individually in the order in which they arrived at the door into the waiting area. During testing a radio was played continuously to minimize the effects of sudden background noises.

Individual pigs entering the testing apparatus were still able to hear and smell their pen mates.

The apparatus, manufactured by Ossendrijver BV (Achterveld, The Netherlands), was a cognitive pig holeboard (Fig. 1a) consisting of a square arena with a 4 × 4 matrix of food bowls. The blue synthetic floor was slatted and the grey synthetic walls (height: 80 cm) had a steel bar across the top. The arena could be entered through any one of the guillotine doors positioned at each of the four side walls, operated from the outside. The entry door for each animal was determined individually by permutations of the numbers 1–4. By voluntarily walking down a small corridor that surrounded the arena, the animals would find an open door and enter the holeboard. The apparatus (arena and corridor) was of the testing area elevated above the floor.

To prevent the animals from locating rewards on the basis of smell, the food rewards (fresh M&M milk chocolates replaced daily) were placed under the false bottom of the food bowls. To prevent the animals from locating the rewards visually, each bowl was covered with a synthetic red ball (JollyBall Dog Toy, diameter: 24 cm, weight: 400 g). The animal would have to lift this ball with its snout, and the reward would then be available for consumption. The design of the apparatus was such that the ball would fall back into place and cover the bowl once the animal retracted its head.

Whenever an animal soiled the apparatus during a trial, the area was cleaned with water before the trial of the next animal. The entire apparatus was rinsed with water daily.

The experiment lasted for a total of 14 weeks. After weaning at 4 weeks of age, the animals were allowed to become habituated to their pen mates and to the new environment in the experimental unit. The animals were gradually exposed to their handlers, the testing room, and the apparatus (two daily sessions, 40 min/pen). Before formal training started, the animals were trained to lift the balls to find rewards and habituated to being alone in the testing apparatus. The group size in the apparatus was therefore gradually decreased (from 8–10 animals to 1 animal) during the 5-week habituation period. After successful habituation, when the animals were 9 weeks of age, formal training started, consisting of three phases (acquisition, first reversal, and second reversal). The animals underwent one trial in the morning and another in the afternoon (spaced trials).

Each of the 9-week old animals was assigned its own configuration of reward holes (4 of 16). Four different configurations were used (Fig. 1b). Each piglet was trained on its particular configuration for 26 spaced trials (13 week days). The entrance door was randomly chosen for each trial. After 26 trials, asymptotic working memory (WM) performance was achieved, and training moved to the next phase.

The first reversal consisted of 38 spaced trials (19 week days). Each individual animal was assigned a changed configuration of rewarded bowls (e.g., a change from A to B–D, Fig. 1b). At this stage, we also wished to investigate the effects of

food restriction on task performance. For this purpose, after 13 days (26 trials) of first reversal training, the feeding schedule of the animals in one of the pens was changed to two feedings a day (see Housing section).

The second reversal was performed for 8 week days. In this phase, each of the pigs received 16 spaced training trials on a new pattern of reward holes (e.g., a change from A to E–H, Fig. 1b). After 10 trials, apple pieces were substituted in place of chocolates for all animals, to look for possible reward-related motivational differences.

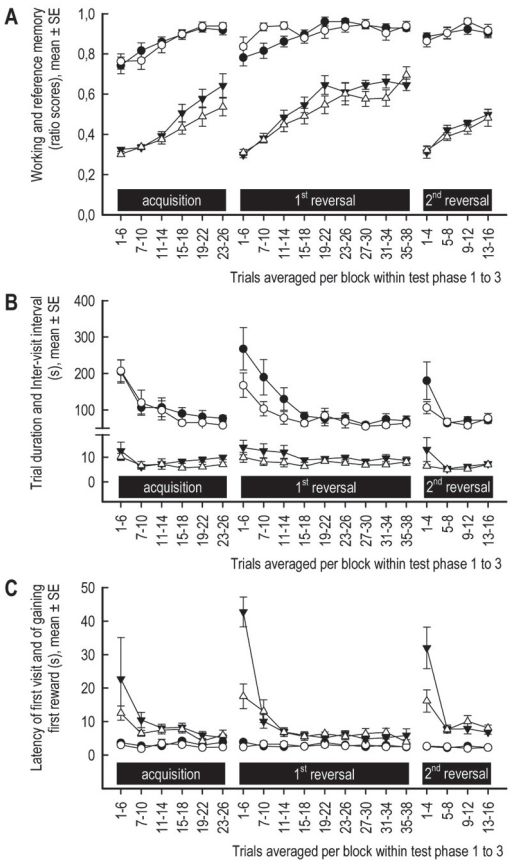


Fig. 1. Schematic holeboard outline. **(a)** Outline of the holeboard used to test spatial memory in piglets. Through the main entrance pigs enter the corridor (width: 40 cm) leading to the open guillotine door (any one of four doors) giving access to the test arena (530 × 530 cm) with 16 symmetrically arranged food bowls (space between bowls: 95 cm, space between wall and bowls: 73 cm, wall height: 80 cm). **(b)** Configurations. One of the four different holeboard configurations (a–d) was assigned to each LBW–NBW pair of siblings in the spatial holeboard task as starting configuration (acquisition). For the first reversal a new configuration was chosen from the patterns a–d, and for the second reversal from patterns e–h). LBW, low-birth-weight; NBW, normal-birth-weight.

Because multiple performance measures were registered, no specific criterion of learning was employed. When WM and RM levels of performance became comparable to those reported by others (Arts et al. 2009), we introduced a reversed configuration.

The number of rewarded (correct) and unrewarded (error) visits, the number of revisits to previously rewarded bowls (errors), the latency (time elapsed) between the first (general) visit and rewarded visit, and the total trial duration were recorded in real time using ANYmaze software (Stoelting, Dublin, Ireland). A visit was

scored when a pig lifted a ball with its snout and an opening between the bowl and the ball became visible. A visit to a previously visited rewarded bowl was scored as a revisit only if at least one other bowl was visited in between. Scoring started when a pig entered the arena with both forelegs. A trial was terminated when a piglet had found and consumed all four food rewards or when 600 s had elapsed, whichever event occurred earlier.

The measures "WM," "RM," "trial duration," "IVIs", and "latency to first (rewarded) hole visit" were calculated and analyzed statistically. Shapiro–Wilk tests for normality of small samples were performed per group for all parameters. This analysis revealed that 65% of all measures were distributed normally. In view of this finding, and because ANOVAs are robust with respect to deviations from normal distribution, we decided to perform all statistical analyses on the untransformed measures.

WM ratio, a measure expressing the percentage of all visits to the set of holes that yielded a food reward, was calculated as (number of rewarded visits)/(number of visits and revisits to the reward set of holes) (Arts et al. 2009).

RM ratio, a measure expressing the number of visits to the reward set of holes as a percentage of the total number of visits to all the holes, was calculated as (number of visits and revisits to the reward set of holes)/(number of visits and revisits to all holes) (Arts et al. 2009).

Trial duration was the time elapsed between entering the holeboard and finding the last reward. If the piglet did not find all of the rewards, this measure was assigned the maximum value (600 s).

IVI, the time per hole visit, was the average time elapsed between two successive hole visits. This measure was calculated as (time elapsed between the first and the last hole visit)/(the number of hole visits – 1).

Latency of first hole visit (the time elapsed up to the first hole visit) was the duration between entering the holeboard and the first contact with a hole, irrespective of whether it belonged to the reward or no-reward set.

Latency to gaining first reward was the time (s) between entering the holeboard and finding the first food reward.

For each of these measures block mean values of four trials each were calculated (with the exception of the first block mean value, which was the average of six trial values).

First, we analyzed whether the procedural adjustments during the course of the holeboard experiment—the change in feeding schedule starting during the first reversal and the reward change during the second reversal—affected holeboard behavior. For this purpose, ANOVAs were performed, with the within-subject (repeated- measures) factor being trial block and the between-subjects

factors being birth weight (LBW vs. NBW), feeding schedule (*ad libitum* vs. twice daily), and type of reward (M&M chocolates vs. pieces of apple). Because these procedural modifications did not differentially affect performance, we excluded these factors from further analyses, and assessed the effects of LBW per phase of the study using a repeated-measures ANOVA with the within-subjects factor being trial block and the between-subjects factor being birth weight supplemented with ANOVAs per trial block.

Results

Spatial Memory

Birth weight did not affect the average WM performance (between-subjects effects, all $F_{1,16} \leq 0.56$, nonsignificant; Table 1 and Fig. 2a). Both groups of piglets improved their WM performance during the three phases of the experiment. Birth weight had an effect on the time required to acquire the WM component of spatial memory during the second pattern of reward holes, i.e., during the first reversal of the experiment (birth-weight × trial block interaction, $F_{8,128} = 2.59$, $P < 0.0117$). During the first few trial blocks of the first reversal, the WM performance of the NBW piglets was better than those of the LBW piglets; the latter group reached the same performance level as their normal weight counterparts only in the fourth block of the first reversal. This suggests that LBW slightly retarded the improvement in WM during this phase of the experiment. Acquisition of the second reversal was not affected by birth weight (Table 1).

Table 1. Behavior of LBW and NBW piglets in a spatial hole-board discrimination task.

		Between subjects effects			**Within subjects effects**					
		Birth-weight (BW)			Trial block (TB)			BW by TB interaction		
Measure	Phase	F	df	p<	F	df	p<	F	df	p<
Working memory	1	0.01	1,16	0.9341	**13.60**	5,80	**0.0001**	0.43	5,80	0.8242
	2	0.56	1,16	0.4639	**6.76**	8,128	**0.0001**	**2.59**	8,128	**0.0117**
	3	0.15	1,16	0.7071	*2.41*	*3,48*	*0.0785*	0.50	3,48	0.6814
Reference memory	1	2.23	1,16	0.1546	**36.63**	5,80	**0.0001**	1.44	5,80	0.2197
	2	0.85	1,16	0.3715	**44.63**	8,128	**0.0001**	1.70	8,128	0.1041
	3	0.48	1,16	0.4964	**23.54**	3,48	**0.0001**	0.43	3,48	0.7355
Trial duration	1	0.21	1,16	0.6520	**15.89**	5,80	**0.0001**	0.31	5,80	0.9032
	2	2.31	1,16	0.1479	**12.56**	8,128	**0.0001**	1.71	8,128	0.1024
	3	1.24	1,16	0.2814	**7.82**	3,48	**0.0002**	1.90	3,48	0.1425
Inter-visit interval	1	1.40	1,16	0.2545	**4.28**	5,80	**0.0014**	0.82	5,80	0.5394
	2	3.32	*1,16*	*0.0872*	**2.43**	8,128	**0.0179**	0.60	8,128	0.7727
	3	2.19	1,16	0.1587	**2.86**	3,48	**0.0463**	1.69	3,48	0.1814
Latency 1st visit	1	2.31	1,16	0.1477	1.66	5,80	0.1528	1.05	5,80	0.3941
	2	0.07	1,16	0.8014	0.63	8,128	0.7476	0.96	8,128	0.4703
	3	0.04	1,16	0.8429	0.54	3,48	0.6565	1.28	3,48	0.2916
Latency 1st reward	1	1.03	1,16	0.3259	**3.20**	5,80	**0.0110**	0.62	5,80	0.6875
	2	1.23	1,16	0.2847	**9.75**	8,128	**0.0001**	**2.82**	8,128	**0.0065**
	3	0.52	1,16	0.4799	**3.75**	3,48	**0.0168**	1.05	3,48	0.3774

The average RM performance in the three phases of the experiment was unaffected by birth weight. Both groups of piglets acquired the different configurations efficiently. The speed of learning was similar in the two groups (Table 1 and Fig. 2a).

The results of repeated-measures ANOVAs for WM, RM, trial duration, IVI, latency until first (rewarded) hole visit are shown. Effects with probabilities <0.05 are shown in bold, whereas marginal effects (0.10 > P > 0.05) are shown in italics. Block: average of four trials (first block, six trials). IVI, intervisit interval; NBW, normal-birth-weight; RM, reference memory; WM, working memory.

Duration Measures

There were no differences between the two groups of piglets as regards trial duration, averaged over all trials. The trial duration decreased across all blocks within the three phases in a similar manner in the LBW and NBW piglet groups (Table 1 and Fig. 2b). A similar picture as for trial duration emerged for the IVIs. Birth weight had no effect on this measure (Table 1 and Fig. 2b).

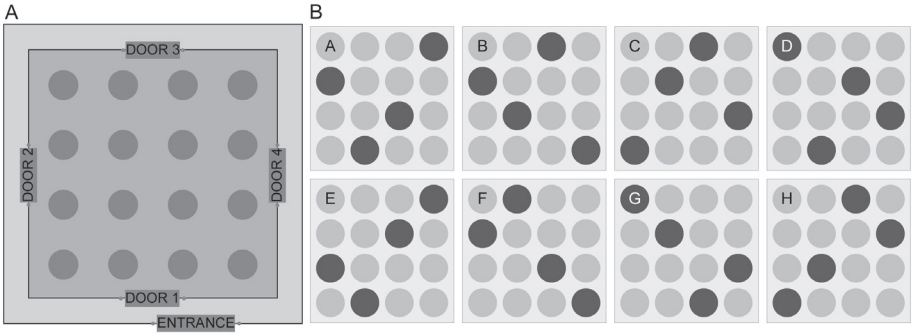


Fig. 2. Behavior of low-birth-weight (LBW, n = 9) and normal-birthweight (NBW, n = 9) piglets in a spatial holeboard task. Means and SEM for the six trial blocks of the acquisition phase, the nine trial blocks of the first reversal, and the four trial blocks of the second reversal are shown for **(a)** WM and RM, **(b)** trial duration and IVI, and **(c)** latency of the first visit and first rewarded visit. **(a)** open circle, WM NBW animals; filled circle, WM LBW animals; open triangle, RM NBW animals; filled triangle, RM LBW animals. **(b)** open circle, latency of NBW animals; filled circle, latency of LBW animals; open triangle, IVI of NBW animals; filled triangle, IVI of LBW animals. **(c)** open circle, 1st visit of NBW animals; filled circle, first visit of LBW animals; open triangle, first reward of NBW animals; filled triangle, first reward of LBW animals. IVI, intervisit interval; RM, reference memory; WM, working memory.

The latency until the first hole was visited, irrespective of whether it belonged to the rewarded set or not, did not change across the blocks of phases I, II, and III, nor did it differ between the two groups of piglets (Table 1 and Fig. 2). The average latency until the piglet gained its first reward was unaffected by birth weight. However, the latency until the first rewarded visit decreased across blocks (Table 1 and Fig. 2c). The decrease was affected by birth weight during the first reversal

(birth weight × trial block interaction, $F_{8,128} = 2.82$, $P < 0.0065$). LBW animals initially showed a relatively high mean latency as compared to NBW animals (LBW: 42.74 s vs. NBW: 17.43 s for the first trial block of the first reversal), but this difference disappeared after the second trial block. Although inspection of Fig. 2c suggests that a similar effect occurred at the beginning of the acquisition, and after switching the animals to the second reversal, this impression was not confirmed statistically.

Discussion

Animals in the LBW group showed transiently retarded acquisition of the first reversed configuration of reward holes. Acquisition of the WM component of spatial memory in the first reversal was slightly retarded for about 12 trials (three blocks) in LBW animals as compared to their NBW siblings, but this difference disappeared with training. In addition, in the LBW group, the latency to finding the first reward was substantially higher in the first trial block after switching to the first reversal.

Our experiment also corroborates previous findings (23) that piglets are able to acquire the cognitive holeboard task. LBW did not affect the acquisition of the first task.

LBW Piglets: An Animal Model of SGA in Humans?

In our study, specific but mild WM deficits were detected in LBW animals whereas RM seemed to be unaffected. Tasks that would be useful for studying WM are not often applied to SGA human infants, because of the lack of developmentally sensitive clinical measures (Baron et al. 2010); however, the few studies that have been performed do show a relationship between LBW and WM performance. It was found that 6-year-old children born with LBW show nonverbal WM deficits, and also planning and cognitive flexibility deficits (Ni et al. 2011). In another study, WM deficits were found in 5.-year old children born with LBW, but these children had all been born preterm (Böhm et al. 2004). The findings in our experiment suggest that the piglets in our experiment can indeed serve as a model for human SGA infants, and the cognitive pig holeboard seems to be a task sensitive enough to detect these mild deficits. Furthermore, the finding that WM deficits occurred whereas RM was spared emphasizes that these two parameters of spatial memory are independent of each other, as has been shown by others (Prickaerts et al. 1999, van der Staay et al. 1990a).

The LBW piglets showed mild, transient deficits when faced with the reversal task. Although these deficits were mild, it is important to consider that we found these differences between groups of piglets that consisted of animal pairs with relatively small birth-weight differences. In pigs, no standard for “underweight” or SGA animals has been defined. This makes it difficult to determine the minimal difference between an NBW or “appropriate for gestational age” animal and an SGA animal. Several standards are in use to define SGA in human neonates. For example ≥2 SD below the mean weight for the nursery of the hospital where the children were born (Pihkala et al. 1989, van der Staay et al. 1990a), or according to the Finnish birth-weight charts (Pihkala et al. 1989). Others prefer

to define as SGA all term infants weighing less than (or equal to) the sex-specific 10th percentile for gestational age at birth (Theodore et al. 2009); this would include all term born children weighing between 1,500 and 2,499 g (Grantham-McGregor et al. 1998) or 2,500 g (Gardner et al. 2003) at birth. Given these variations in the definition of SGA in humans, it is possible that more stringent criteria for definitions of LBW and NBW in piglets are needed. In our experiment, an LBW piglet was defined as one weighing at least 1 SD below the litter average. On the third day after birth, the average weight difference between pairs of siblings was 549 g (SD 272 g, minimal difference within pairs 300 g, maximal difference 980 g) with absolute weights varying between 960 and 2,525 g. In the future, this type of research would require testing pairs of siblings with larger birth-weight differences, in accordance with the stricter definition in human research.

All of the piglets had been delivered naturally without complications. However, the possibility that hypoxic–ischemic episodes may have occurred during delivery could not be entirely excluded. We did not observe motor or other deficits in any of the animals, and therefore, the possibility of hypoxia is expected to be limited. In future studies it would be advantageous to control the delivery process to prevent hypoxia, so as to avoid any interference from extraneous factors other than those related to SGA and growth retardation.

Motivation and Response Flexibility

Hand in hand with the difficulty in reversal learning comes an increased latency to finding the first rewarded bowl. Indeed, LBW animals did need more time as compared to their NBW siblings in the first trials after switching to the new reward pattern. Although LBW animals showed a retarded acquisition of the first reversal, they completed a trial in the same time period as their NBW siblings (trial duration). As with trial duration, no differences were found in IVI, indicating that LBW and NBW animals are equally motivated and physically able to perform the cognitive holeboard task.

The initial latency until an animal found its first reward differed between LBW and NBW animals during the initial stage of acquiring the first reversal. As the latency to reach the first bowl in general (rewarded or unrewarded) did not differ between groups, we hypothesize that the LBW animals showed reduced response flexibility or behavioral inhibition when confronted with an unexpected change in the test environment, which would have become noticeable when the first expected reward was not found. This hypothesis is in line with the finding that growth-retarded children with LBW appear to be less adaptive to changing test conditions. These children also showed difficulties in producing correct strategy solutions (Leitner et al. 2005). Therefore future studies should include measurements for “flexibility to change” and “strategy-related differences.” The cognitive pig holeboard is a suitable apparatus for collecting such measures.

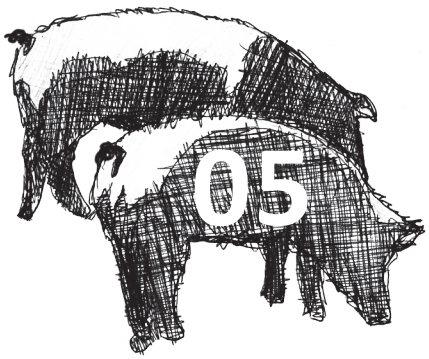
Modifications of the Holeboard Apparatus and Procedure

Compared with the setup used by others (Arts et al. 2009) our holeboard apparatus and training procedure had some modifications. We provided the holeboard apparatus with four entries instead of one, thereby reducing the likelihood that the

task is solved in a nonspatial manner (van der Staay et al. 1990b). Randomizing entry positions across trials did not seem to influence learning speed. On average, the WM and RM scores reported in a previous pig holeboard study (Arts et al. 2009) were reached in our study in ~25 trials, thereby indicating that the cognitive pig holeboard yields stable and repeatable results.

Conclusion

Using a cognitive holeboard task, we found some evidence to suggest that piglets born with LBW have more difficulty in switching from one learned configuration to a new one as compared to their NBW siblings. Further research is required to clarify to what extent LBW influences cognitive performance in pigs. The holeboard task proved to be suitable for testing spatial discrimination learning in pigs. However, further validation of holeboard tasks in pigs is needed to determine the level of difficulty and sensitivity necessary to reveal small differences between groups. By fine-tuning the definition for LBW/SGA in piglets, and by adding additional behavioral, physiological, and brain measures to these types of studies, this promising model for long-term cognitive effects of LBW in humans can be refined and extended.



Performance of conventional pigs and Göttingen miniature pigs in a spatial holeboard task: effects of the putative muscarinic cognition impairer biperiden

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Abstract

Background: The pig is emerging as a model species that bridges the gap between rodents and humans in research. In particular, the miniature pig (referred to hereafter as the minipig) is increasingly being used as non-rodent species in pharmacological and toxicological studies. However, there is as yet a lack of validated behavioral tests for pigs, although there is evidence that the spatial holeboard task can be used to assess the working and reference memory of pigs. In the present study, we compared the learning performance of commercial pigs and Göttingen minipigs in a holeboard task.

Methods: Biperiden, a muscarinic M1 receptor blocker, is used to induce impairments in cognitive function in animal research. The two groups of pigs were treated orally with increasing doses of biperiden (0.05 – 20 $mg.kg^{-1}$) after they had reached asymptotic performance in the holeboard task.

Results: Both the conventional pigs and the Göttingen minipigs learned the holeboard task, reaching nearly errorless asymptotic working and reference memory performance within approximately 100 acquisition trials. Biperiden treatment affected reference, but not working, memory, increasing trial duration and the latency to first hole visit at doses ≥ 5 $mg.kg^{-1}$.

Conclusion: Both pig breeds learned the holeboard task and had a comparable performance. Biperiden had only a minor effect on holeboard performance overall, and mainly on reference memory performance. The effectiveness needs to be evaluated further before definitive conclusions can be drawn about the ability of this potential cognition impairer in pigs.

Keywords: Working memory – Reference memory – Animal model – Holeboard task – Spatial learning task – Biperiden

Introduction

Although most preclinical studies in neurosciences are performed using rodents, in particular mice, the pig is increasingly being used as a model species because it can bridge the anatomical/physiological gap between rodents and humans (Bollen et al. 2007, Swindle et al. 2012, Walters et al. 2011). In particular, the miniature pig (referred to hereafter as the minipig) is gaining popularity as laboratory animal in pharmacological and toxicological studies (van der Laan et al. 2010); however, pigs have not been used extensively in behavioral studies, mainly because there are few validated behavioral tests for pigs. Recent systematic reviews have found pigs able to acquire a broad range of learning and memory tasks (Gieling et al. 2011, Kornum and Knudsen. 2011), such as the holeboard task (Arts et al. 2009, Gieling et al. 2012).

The holeboard is a food-rewarded maze, where bait can be found in different places, and the animal is free to visit these places in whatever order it chooses. Once an animal has visited a place and consumed the food, that place is not rebaited during a trial and thus return visits to the same location are not reinforced (reviewed in van der Staay et al. (2012)). If food can only be found in a subset of potential sites, then two memory components can be distinguished and measured simultaneously: spatial working memory (WM) and reference memory (RM) (Olton et al. 1979). WM holds information that is relevant only within a specific trial, such as a list of locations that have recently been visited/explored. An animal thus must process the temporal context associated with an event – "what happened, and when did it happen" – and keep in mind which locations have already been visited, in order to efficiently deal with the spatial WM component of a task (Bannerman and Sprengel. 2007). The information is transiently held in memory until the specific trial has been completed and is of no value for performing the next trial. RM holds information about the solution of the spatial holeboard discrimination task, such as the localization of the food and the actions necessary to get the bait (Dudchenko. 2004), for example, lifting up a ball covering each food bowl with the snout (Gieling et al. 2012). RM thus stores the general rules of a task. It retains relevance across many trials and is thus trial independent, but learning task specific. Little is known about the ability of Göttingen minipigs to learn the holeboard task. Manton (2010) tested minipigs in a holeboard at the ages of approximately 59, 88, and 93 days, on each occasion over 2 consecutive days with 3 training trials per day, and found the animals to have a very poor WM and RM performance, with RM performance hardly exceeding chance level.

Central cholinergic neurotransmission appears to be involved in spatial learning and memory processes (Deiana et al. 2011). Of the five known muscarinic receptor subtypes, M1, M2, M4, and M5 are found in the human brain. M1, M2, and M4 receptors are abundant in the hippocampus and cortex, areas that that involved in learning and memory. Blockade of these receptors thus is expected to induce cognitive deficits (de Leon. 2011). The non-selective muscarinic receptor antagonist scopolamine is used to induce cognitive impairments in animal models of disorders characterized by cognitive dysfunctions (Klinkenberg and Blokland. 2010), such as Alzheimer's disease. Bouger and van der Staay (2005) showed

that administration of scopolamine (or the non-competitive NMDA antagonist MK-801) in well-trained rats transiently, but consistently, impaired WM and RM in a conefield, a variant of the holeboard. Recently, it has been suggested that biperiden, a muscarinic M1 receptor antagonist (de Leon. 2011), may be better suited to induce cognitive deficits in animal experiments (Klinkenberg and Blokland. 2011). Biperiden (manufactured as Akineton by BASF/Knoll Pharma, New Jersey, USA) is generally used as an antiparkinsonian agent in humans. In humans, the drug has a poor availability of only 13% after oral administration; its bioavailability after systemic administration is 33% (+/- 5%). The tmax of biperiden is 0.5–1.5 h and the t½ is 21 h (+/- 3.1 h). (Grimaldi et al. 1986) Biperiden appears to be tolerated in high dosages before overt and lethal toxicity occurs: an oral LD50 of 750 $mg.kg^{-1}$ has been reported for rats and 340 $mg.kg^{-1}$ for dogs (Hanna. 1960). Common observable side effects and neuropsychiatric signs include dry mouth, drowsiness, agitation, anxiety, hyperactivity, ataxia, and loss of memory (U.S. National Library of Medicine 2006).

In line with the expectation that M1 receptor blockade would impair cognition, biperiden-induced cognitive deficits have been observed in different species, including humans (Wezenberg et al. 2005). In humans, motor learning as well as visuospatial processes were impaired after oral administration of 2 mg biperiden (Wezenberg et al. 2005). Silver and Geraisy (1995) observed that biperiden (2 mg twice daily) given to schizophrenic patients impaired performance on the Benton Visual Retention Test and the visual subscale of the Wechsler Memory Scale. Similarly, Liang et al. (2010) showed that patients with schizophrenia treated twice daily with 2 mg biperiden had impaired cognitive functions, tested using the Mini Mental State Examination (MMSE). In rats, biperiden has been found to impair responding at 10 $mg.kg^{-1}$ and to impair short-term memory in a series of operant learning and memory tasks (Grimaldi et al. 1986), but it did not affect food motivation or attention. In another study, biperiden (4, 8, or 16 $mg.kg^{-1}$, injected intraperitoneally) delayed consolidation in a passive avoidance task (Roldán et al. 1997).

As scopolamine is a non-selective muscarinic receptor antagonist, it is difficult to establish whether its behavioral effects are mediated centrally (cognitive) or peripherally (side effect). Biperiden might be a better choice because it is more selective. Moreover, scopolamine is usually administered intraperitoneally or intramuscularly (Klinkenberg and Blokland. 2010), but in pigs these routes of administration might be unduly stressful, which could interfere with learning (Schwabe et al. 2012). In contrast, biperiden is administered orally. Oral administration in food is the least stressful and preferred way to administer drugs to pigs provided that the drug-containing food does not induce food aversion and is palatable (Ellegaard et al. 2010). As we would like to know whether Göttingen minipigs are suitable for studying cognitive function, we tested whether 1) conventional pigs and age-matched Göttingen minipigs learn the holeboard task at a similar speed and to a similar asymptotic performance level, and 2) whether orally administered biperiden transiently impairs spatial memory in pigs. We expected that the Göttingen minipigs would be able to learn the holeboard task as good as conventional pigs do and that biperiden would impair cognition in

both pig lines, as was found earlier with rats (Klinkenberg and Blokland. 2011). We expect biperiden to be an interesting alternative for scopolamine for inducing cognitive impairment.

Material and methods

Ethical approval

The experiments were reviewed and approved by the local ethics committee (DEC, dierexperimentencommissie) and were conducted in accordance with the recommendations of the EU directive 86/609/EEC. All efforts were made to minimize the number of animals used and to avoid suffering.

Animals

Eight female Göttingen miniature pigs (supplier: Ellegaard Göttingen Minipigs A/S, Dalmose, Denmark) and 8 female piglets [Duroc X (Fin X York)] born at the pig-breeding farm of Utrecht University were used. Healthy piglets from different litters were selected after weaning and were moved in a covered trolley to our experimental facility when they were 4–6 weeks old. The minipigs were transported from Denmark to the Netherlands in a climate-controlled minivan.

Housing

The pigs were housed together per breed in two adjacent identical pens of 20 m^2, situated in a large, naturally ventilated and lit stable. The pens had a concrete floor, covered with straw bedding; drinking water was provided *ad libitum*. A covered piglet nest, a play ball, and chewing sticks were provided per pen. In addition, a heating mat (25°C) covered with straw was installed in the nest of the Göttingen minipigs. All pigs were fed twice a day, once in the early morning (1/3 of their daily feed ration in the morning, about 1 hour before testing started) and once in the late afternoon (2/3 of their daily feed ration after completion of daily holeboard training). The Göttingen minipigs were fed on a diet according to the recommendations of the breeder. All pigs were weighed at least once a week.

Testing room

The testing equipment was located in the room next to the pens. The testing room consisted of a corridor, a waiting area, and the holeboard apparatus. All pigs from one group were walked down the corridor and entered the straw- and toy-enriched waiting area (11.5 m^2, *ad libitum* access to drinking water) of the testing apparatus. Then the experimenter let one pig into the testing apparatus. After testing, the pig returned to the waiting area.

Drug

Akineton tablets (containing 2 mg Biperiden; producer: Abbott Laboratories) were crushed and mixed with conventional pig food, honey (to make it more palatable), and some water into a ball.

Apparatus

The holeboard consisted of a square arena measuring 530 by 530 cm, with a 4x4 matrix of food bowls (for a schematic overview of the apparatus see Gieling et al. (2012)). The blue synthetic floor was slatted and the gray synthetic walls (height:

80 cm) had a steel bar on top (at a height of 100 cm). The arena had four entries (one on each side) through guillotine doors that were operated from the outside by the experimenter using pulley strings. By walking down a small corridor (width: 40 cm) surrounding the entire arena, the animals found the opened door and entered the holeboard on their own initiative. The apparatus (arena and corridor) was elevated above the floor. The testing room provided adequate extra-maze cues.

To prevent the pigs from locating rewards based on smell, all food bowls had false bottoms under which fresh rewards (M&M milk chocolates®) were placed daily. To prevent the animals from locating the rewards visually, each bowl was covered with a synthetic red ball (Jolly Ball Dog Toy, diameter: 24 cm, weight: 400 g). The pigs could get at the reward by lifting the ball with their snout; the ball rolled back on the bowl as soon as the pig withdrew its head. The apparatus was cleaned with water before the next trial, and the entire apparatus was rinsed with water daily after use.

Habituation

When the pigs were approximately 10 weeks old, they were gradually exposed to their handlers, the testing room, and the apparatus, as described by Gieling et al. (2012). During the first week of habituation, the pigs were allowed to get used to the experimenters and the waiting area, and from the second week they were habituated to the holeboard. The pigs had four daily sessions of 20 minutes per pen. The animals were first habituated to the holeboard in groups of 8 pigs, then in groups of 4 pigs, and lastly in groups of 2 pigs. During the habituation period, all food bowls contained M&Ms. Individual pigs were habituated to the holeboard during four trials/day on 10 successive working days until the pig had found all rewards or 10 minutes had passed, whichever event occurred first. The next working day, training was started.

Acquisition

When the pigs were about 13 weeks old, formal training in the holeboard started. Each animal was assigned its own configuration of four rewarded holes. Four different configurations were used (determined using the rule outlined in Fig. 4K in van der Staay et al. (2012): a basic configuration (Fig. 4E in van der Staay et al. (2012)) and its three rotations (90, 180, and 270 degrees). In this way, all sixteen holes were rewarded equally often. The entrance door was randomly chosen for each trial. Each pig received 2 acquisition sessions of 2 trials each day (1 session in the morning and 1 session in the afternoon) for the first 13 working days (i.e. 26 sessions). Then, from working day 14 to 40, each daily session consisted of 2 massed trials. The acquisition phase consisted of 104 trials. Testing was never performed during weekends.

Data collection

Data collection was automated. Each food bowl was equipped with a hidden sensor. If the ball (which was fitted with a magnet) on top of the bowl was lifted, a signal was broken and sent to an interface (LabJack) and stored on a Personal Computer (OS: Windows XP), using the custom made software 'Experiment control for University Utrecht' (Blinq Systems, Delft, The Netherlands).

A trial ended automatically after an animal had found all rewards or 10 minutes had elapsed.

Drug treatment

After they reached asymptotic performance, all pigs were treated with increasing doses of biperiden (0.05 mg.kg^{-1}, 0.15, 1.5, 5, 15 and 20 mg.kg^{-1}; the lowest dose corresponds to the therapeutic dose in humans) administered orally 1.5 hours before holeboard testing started. As in the training trials, testing consisted of 2 trials in close succession. At 8:30, the first pig was given its ball of food containing biperiden in a food bowl (to prevent spoiling), followed at 15-minute intervals by the other pigs in random order. This was order in which the pigs were tested. Pigs were given the rest of their morning ration of feed 30 minutes after administration of the two lowest doses of biperiden, to ensure that their performance was not altered by hunger. This was not necessary for the higher doses, because the drug-feed mixture contained the pigs' normal feed ration. Between each drug-testing session, there was a washout period of at least 2 days, based on the t½ of 18.4–24.3 hours of biperiden in humans and rodents. The pigs continued to train during the wash-out periods. Biperiden was tested in sessions 22 (0.05 mg.kg^{-1}), 32 (0.15 mg.kg^{-1}), 36 (1.5 mg.kg^{-1}), 39 (5 mg.kg^{-1}), 41 (15 mg.kg^{-1}), and 44 (20 mg.kg^{-1}).

Drug-induced side effects

The behavioral side effects of biperiden (Gerretsen and Pollock. 2011) were registered by two researchers while the pigs were in the waiting area. Behavioral sedation was scored when an animal lay down 1 minute or longer. Dry mouth (shown by yawning-like behavior) and dry cough were scored if these behaviors were observed more than twice.

Statistical analysis

WM and RM are expressed as ratios (van der Staay et al. 2012). WM was defined as the number of rewarded visits divided by the number of visits to the baited set of holes. This ratio reflects the ability of the animals to avoid re-visits to baited holes during a trial. RM was defined as the number of visits to the baited set of holes divided by the number of visits to all holes. This ratio reflects the ability of animals to discriminate between baited and unbaited holes.

To analyze differences in the speed of holeboard task acquisition by the two groups of pigs, the means of blocks of 4 trials (1 testing day) were calculated. Changes in WM, RM, trial duration, and latency to first rewarded hole visit in the course of training were assessed by an analysis of variance (ANOVA) with the between subjects factor Pig breed (conventional pigs vs. Göttingen minipigs) and the within subjects (repeated measures) factor Blocks of Trials (SAS GLM procedure, SAS Institute, Cary, NC, USA). To analyze the effect of acute challenge with biperiden, means were calculated for both drug and drug-free sessions. Both session means consisted of two successive trials. First we tested whether the drug-free sessions differed from each other (repeated measures ANOVA) to decide whether an overall drug-free session mean could be used or whether separate sessions should be analyzed. To assess whether biperiden affects holeboard behavior, and whether this effect is different for the two pig breeds, we performed a Pig breed (Conventional

pigs vs. Göttingen minipigs) by Doses (0.05, 0.15, 1.5, 5, and 15 mg.kg^{-1} Biperiden) by Sessions (Control session preceding Biperiden treatment vs. Biperiden session) ANOVA with repeated measures on the second and third factors. Unfortunately, one of the conventional pigs did not eat the 15 mg.kg^{-1} dose of biperiden, so the data for this pig were omitted from the repeated measures analysis. Because the conventional pigs did not eat the entire of 20 mg.kg^{-1} dose of biperiden, only the doses up to 15 mg.kg^{-1} were considered for comparisons between pig breeds. In addition, we analyzed the effects of biperiden in the Göttingen minipigs by a Doses by Sessions repeated measures ANOVA for all doses tested (i.e. including the 20 mg.kg^{-1} biperiden dose). An alpha of < 0.05 was considered significant.

Results

Acquisition of the holeboard task

Working memory

WM performance (see Fig. 1A) was similar in the two groups of pigs ($F_{1,14} = 0.02$, $p = 0.8939$) and improved with training (Blocks of trials: $F_{25,350} = 7.41$, $p < 0.0001$) similarly in the two groups of pigs (Pig breed X Blocks of trials interaction: $F_{25,350} = 1.10$, $p = 0.3607$).

Reference memory

The Göttingen minipigs tended to have a poorer RM (see Fig. 1B) than the conventional pigs ($F_{1,14} = 4.02$, $p = 0.0646$). RM performance improved across blocks of trials (Blocks of trials: $F_{25,350} = 104.43$, $p < 0.0001$), and while learning appeared to be slightly delayed in the Göttingen minipigs (Pig breed X Blocks of trials interaction: $F_{25,350} = 1.44$, $p = 0.0811$), both groups of pigs ultimately had a similar level of performance.

Trial duration

The two groups of pigs completed the trials at a similar speed across all trial blocks ($F_{1,14} = 1.31$, $p = 0.2708$), and trials became shorter in the course of learning (Blocks of trials: $F_{25,350} = 6.51$, $p < .0001$) in both groups of pigs (Pig breed X Blocks of trials interaction: $F_{25,350} = 0.66$, $p = 0.8910$). This is shown in Fig. 1C.

Latency to first rewarded hole visit

As is shown in Fig. 1D, the conventional pigs gained their first reward faster than the Göttingen minipigs ($F_{1,14} = 12.39$, $p < 0.0034$), but pigs in both groups became quicker in finding the food reward in the course of training (Blocks of trials: $F_{25,350} = 12.61$, $p < 0.0001$) (Pig breed X Blocks of trials interaction: $F_{25,350} = 1.67$, $p = 0.0252$). Whereas the Göttingen minipigs needed more time to find the first reward than the conventional pigs during the first block of four trials ($t_{14} = -2.19$, $p = 0.0462$), the performance of both lines was similar during the last block of four trials ($t_{14} = -1.64$, $p = 0.1241$).

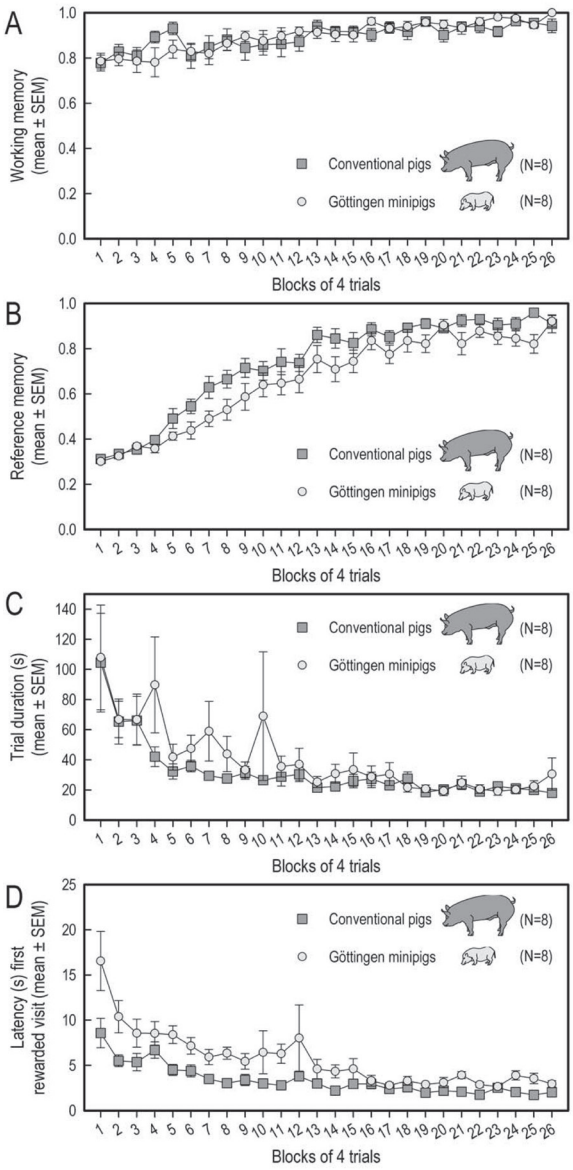

Fig. 1 Learning of a spatial holeboard discrimination task by conventional pigs and Göttingen minipigs.
The working memory performance (panel A) and the reference memory performance (panel B), the trial duration (panel C) and the latency to first rewarded hole visit (panel D) are depicted as means and standard errors of the mean (SEM) of 26 successive blocks of 4 trials each.

Effects of biperiden

Side effects

Most pigs showed signs of a dry mouth (xerostomia) and mild behavioral sedation (lying down for longer than normal in the waiting area) with the higher doses of biperiden ($\geq$ 15 mg.kg^{-1}) (Fig. 2).

Control (drug-free) sessions

Performance during the drug-free sessions that preceded test days did not change, with the exception of RM ($F_{5,70}$ =2.92, p = 0.0190). Contrast variables showed that for RM only sessions 1 and 2 ($F_{1,14}$ = 5.11, p = 0.0403) and 3 and 4 ($F_{1,14}$ = 9.29,

p = 0.0087) differed from each other. On the basis of these results, we decided to compare a treatment session with its own preceding drug-free session and not with the average of all drug free sessions preceding a treatment session.

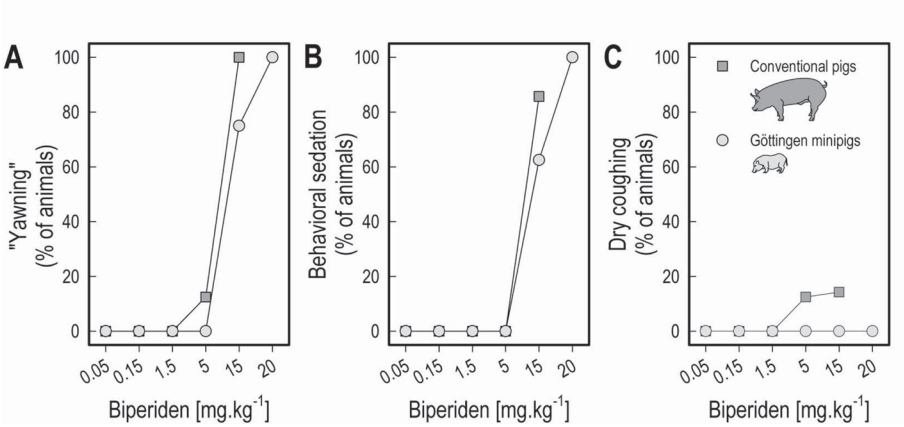

Fig. 2 Side effects of biperiden at different doses. Panel A: percentage of animals with signs of dry mouth ("yawning"). **Panel B:** percentage of animals showing signs of behavioural sedation. **Panel C:** percentage of animals with dry cough. As the conventional pigs did not eat all of the 20 mg/kg^{-1} dose, the side effects of this dose are excluded for this group (Note: legend in panel C also applies to panels A and B).

Working memory

The WM performance (see Fig. 3A) of the two groups of pigs, averaged over all doses up to 15 mg.kg^{-1} and sessions, was not significantly different ($F_{1,13}$ = 0.00, p = 0.9595), and biperiden did not affect WM (Dose: $F_{4,52}$ = 0.03, p = 0.9979); Pig breed X Dose interaction: $F_{4,52}$ = 0.89, p = 0.4742; Session: $F_{1,13}$ = 0.62, p = 0.4446.; Pig breed X Sessions interaction: $F_{1,13}$ = 0.30, p = 0.5952; Dose X Sessions interaction: $F_{4,52}$ = 1.55, p = 0.2012; Pig breed X Dose X Sessions interaction: $F_{4,52}$ = 0.52, p = 0.7189). However, analysis of the data for all biperiden doses tested (0.05–20 mg.kg^{-1}) revealed that biperiden affected WM in the minipigs (Doses X Sessions interaction: $F_{5,35}$ = 3.25, p = 0.0163; Doses: $F_{5,35}$ = 0.46, p = 0.8020; Sessions: $F_{1,7}$ = 0.36, p = 0.5666).

Reference memory

The RM performance (see Fig. 3B) of the two groups of pigs was similar, averaged over all doses and sessions ($F_{1,13}$ = 2.00, p = 0.1807), and biperiden treatment affected RM performance (Dose: $F_{4,52}$ = 3.73, p = 0.0096) similarly in the two groups (Pig breed X Dose interaction: $F_{4,52}$ = 0.69, p = 0.5995; Session: $F_{1,13}$ = 4.66, p = 0.0501; Pig breed X Sessions interaction: $F_{1,13}$ = 0.44, p = 0.5172; Dose X Sessions interaction: $F_{4,52}$ = 2.53, p = 0.0511; Pig breed X Dose X Sessions interaction: $F_{4,52}$ = 1.17, p = 0.3339). Note that the Sessions effect, i.e. the overall performance in the control sessions vs. the overall performance in the drug treatment sessions, had an associated probability close to 0.05. This suggests that, on average, the pigs performed worse in the biperiden sessions. The marginal Dose X Sessions interaction suggests that the impairment induced by biperiden was dose

dependent. Analyses revealed a marginal dose-related effect of biperiden in the minipigs (Doses X Sessions interaction: $F_{5,35} = 2.21$, $p = 0.0748$, Doses: $F_{3,35} = 3.64$, $p = 0.0093$; Sessions: $F_{1,7} = 4.21$, $p = 0.0792$), with higher doses (except 15 $mg.kg^{-1}$) appearing to decrease RM performance (see Table 1).

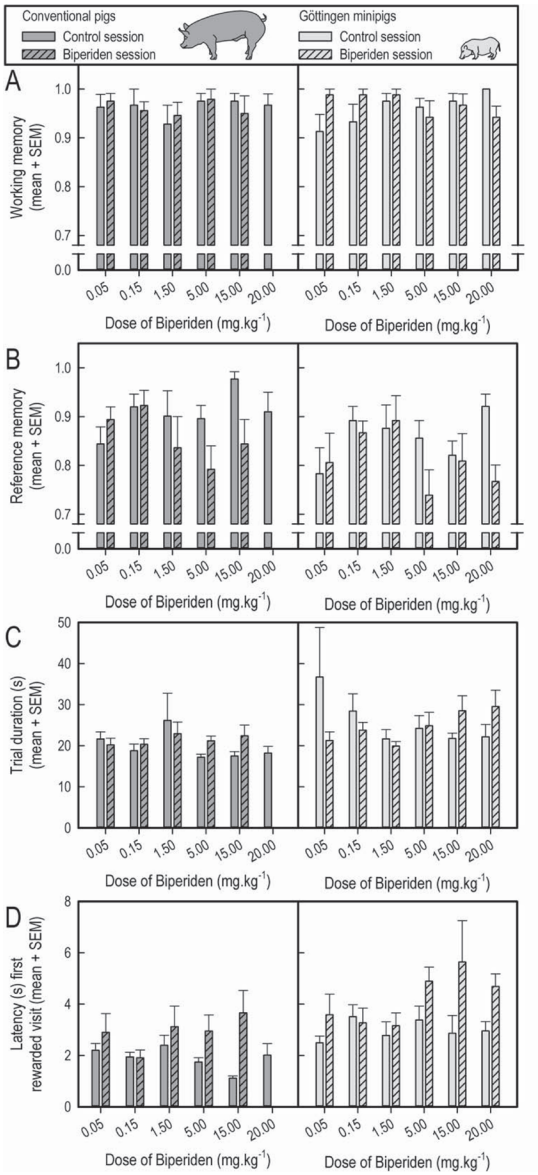

Fig. 3. Effects of oral administration of increasing doses of biperiden on working memory (A) and reference memory performance (B), trial duration (C), and latency to first rewarded hole visit (D) of conventional pigs and Göttingen minipigs. The means ± SEM of the drug-free day preceding treatment and of the day of biperiden treatment are depicted. None of the conventional pigs consumed the entire 20 $mg.kg^{-1}$ dose. Consequently, these data were not analyzed, and only the data of the Göttingen minipigs are shown for the highest dose of Biperiden.

Table 1. Biperiden effects per pig line and dose of Biperiden. One-sample t-statistics and the associated p-values of the difference scores between the control sessions preceding treatment and the corresponding treatment sessions are tabulated. Abbreviation: n.t. not tested, because none of the conventional pigs consumed the entire dose of 20 mg.kg^{-1}. p-values < 0.05 are printed bold and italicized, p-values between 0.05 and 0.1 (marginal effects) are italicized.

Biperiden (mg.kg^{1} body mass, p.o.)	Conventional pigs		Göttingen minipigs		Conventional pigs		Göttingen minipigs	
	Working memory				Reference memory			
0.05	t_8= 0.36	p=0.7318	t_8= 2.05	*p=0.0796*	t_8= 1.18	p=0.2765	t_8= 0.34	p=0.7425
0.15	t_8=-0.24	p=0.8157	t_8= 1.63	p=0.1477	t_8= 0.09	p=0.9332	t_8=-0.66	p=0.5277
1.5	t_8= 0.39	p=0.7055	t_8= 0.55	p=0.5983	t_8=-1.43	p=0.1966	t_8= 0.27	p=0.7986
5	t_8= 0.14	p=0.8916	t_8=-0.62	p=0.5577	t_8=-2.96	***p=0.0212***	t_8=-4.75	***p=0.0021***
15	t_7=-0.46	p=0.6585	t_8=-0.41	p=0.6975	t_7=-2.41	*p=0.0528*	t_8=-0.22	p=0.8316
20	n.t.		t_8=-2.50	***p=0.0412***	n.t.		t_8=-3.55	***p=0.0094***
	Trial duration				Latency to first rewarded hole visit			
0.05	t_8=-0.81	p=0.4427	t_8=-1.31	p=0.2325	t_8=-1.00	p=0.3519	t_8=-1.25	p=0.2522
0.15	t_8= 0.89	p=0.4032	t_8=-1.45	p=0.1895	t_8= 0.08	p=0.9375	t_8= 0.29	p=0.7797
1.5	t_8=-0.49	p=0.6358	t_8=-0.78	p=0.4612	t_8=-1.18	p=0.2749	t_8=-0.74	p=0.4831
5	t_8= 4.27	***p=0.0037***	t_8= 0.21	p=0.8401	t_8=-1.63	p=0.1476	t_8=-1.98	*p=0.0876*
15	t_7= 1.55	p=0.1725	t_8= 2.05	*p=0.0792*	t_7=-3.00	***p=0.0241***	t_8=-1.57	p=0.1614
20	n.t.		t_8= 1.98	*p=0.0887*	n.t.		t_8=-5.11	***p=0.0014***

Trial duration

Averaged over all doses and sessions biperiden did not differentially affect trial duration in the two groups (Doses: $F_{4,52}$ = 0.43, p = 0.7834; Pig breed X Doses interaction: $F_{4,52}$ = 1.00, p = 0.4143; Pig breed X Doses X Sessions interaction: $F_{4,52}$ = 0.88, p = 0.4842). Trial duration tended to increase with increasing biperiden dose (Sessions: $F_{1,13}$ = 0.35, p = 0.5646; Dose X Sessions interaction: $F_{4,52}$ = 2.23, p = 0.0784; Pig breed lines X Sessions interaction: $F_{1,13}$ = 1.17, p = 0.2997). Analysis of data for the minipigs revealed that the higher doses of biperiden increased trial duration more than did the lower doses (Doses X Sessions interaction: $F_{5,35}$ = 3.34, p = 0.0143; Doses: $F_{5,35}$ = 0.78, p = 0.5712; Sessions: $F_{1,7}$ = 0.00, p = 0.9525). Trial duration is shown in Fig. 3C.

Latency to first rewarded hole visit

As is shown in Fig. 3D, the minipigs took longer to find the first food reward than did the conventional pigs ($F_{1,13}$ = 8.05, p = 0.0140), but biperiden did not affect the time it took in either group (Dose: $F_{4,52}$ = 1.08, p = 0.3772; Pig breed X Dose interaction: $F_{4,52}$ = 1.42, p = 0.2416). However, across all doses tested, the pigs took longer to find the first food reward when they were treated with biperiden than in the preceding control session (Session: $F_{1,13}$ = 14.55, p = 0.0021), with a marginal Dose X Sessions interaction suggesting that the higher, rather than lower, doses of biperiden tended to increase the time it took pigs to find the first food reward ($F_{4,52}$ = 2.54; p = 0.0508). This effect was similar for the two groups of pigs (Pig breed X Sessions interaction: $F_{1,13}$ = 0.01, p = 0.9236; Pig breed X Dose X Sessions interaction: $F_{4,52}$ = 0.04, p = 0.9968). Analysis of

the data for the minipigs confirmed that this effect of biperiden on the time taken to find the food reward was not dose related (Dose X Sessions interaction: $F_{5,35} = 1.16$, $p = 0.3476$).

Discussion

Both the conventional pigs and the Göttingen minipigs learned the holeboard task. This finding corroborates and extends earlier studies by Arts et al. (2009) and Gieling et al. (2012) and confirms our hypothesis. After intensive training (about 100 trials), the pigs reached nearly errorless asymptotic WM and RM performance. Moreover, all pigs had a higher level of performance than we have ever seen in rats, even after more that 400 training trials (Bouger and van der Staay. 2005). The holeboard performance and motivation of minipigs were not different from those of conventional pigs, showing that both types of pig can be used in cognitive research. This is in marked contrast to the conclusion drawn by Downes (2012), based on a study by Manton (2010), that "the results of testing for learning and memory in minipigs were equivocal and ultimately disappointing". We found that minipigs could acquire the spatial holeboard discrimination task, suggesting that Downes' conclusions concerning testing the learning and memory capacity of minipigs, using the holeboard task are premature.

The cholinergic system is involved in spatial discrimination learning (Deiana et al. 2011), and holeboard-type tasks are sensitive to manipulation of cholinergic neurotransmission with so-called cognition enhancers or cognition impairers (for a recent review see van der Staay et al. (2012)). Biperiden, an M1 receptor antagonist, is suggested to act as a cognition impairer (Klinkenberg and Blokland. 2010, Klinkenberg and Blokland. 2011, Kornum and Knudsen. 2011), but we found only marginal effects on RM and WM at very high oral doses (5 to 20 $mg.kg^{-1}$). The WM performance of the conventional pigs appeared to be unaffected by biperiden, whereas the drug appeared to differentially affect WM performance in the minipigs, with the lowest dose of biperiden marginally improving WM performance and the highest dose marginally impairing WM performance (see Table 1). On the basis of the effects of the cognition impairers scopolamine and MK-108 in well-trained rats (Bouger and van der Staay. 2005), we expected that biperiden would transiently affect WM and RM performance. The lowest dose of biperiden that impaired RM in both groups of pigs was 5 $mg.kg^{-1}$, which is in the dose range that was found to affect cognition in rats (Klinkenberg and Blokland. 2011). Thus although we had expected that biperiden would transiently impair spatial memory in conventional pigs and age-matched minipigs, once they have learned the cognitive holeboard task, we cannot unambiguously conclude that this is the case. We can conclude that the effects found on RM are comparable for both breeds, although the highest dose could not be tested in conventional pigs.

It may be difficult to impair memory performance once a task has been learned to almost perfection, but previous studies with the conefield task, a variant of the holeboard task, have shown that the near-maximal, asymptotic WM and RM performance of rats could be decreased with the cognition impairers scopolamine and MK-801. In the drug-free session following each of the scopolamine or MK-801 sessions, the WM and RM performance returned to control level (Bouger

and van der Staay. 2005). Although the rats reached an asymptotic performance, they did not reach the maximum performance possible (ceiling level). In the current study, the peak performance of the pigs was close to the maximum performance level possible. In order to detect cognition-impairing effects, it might be appropriate to give biperiden earlier, before pigs reach an asymptotic level of performance, or the task could be made more difficult (e.g. hide 5 instead of 4 rewards). This is supported by the observation that while the performance of laboratory animals in the conefield task can be altered by cognition impairers (Bouger and van der Staay. 2005), performance in other spatial learning and memory tasks, such as the Morris water escape task, is unresponsive to the effects of cognition impairers once the task has been learned to an asymptotic level (van der Staay et al. 2011). In a study involving a well-learned operant task and rats (Liu. 1996), biperiden at doses of 0.25 and 0.5 $mg.kg^{-1}$ increased the number of non-reinforced responses and decreased the number of reinforcements obtained. Doses exceeding 0.5 $mg.kg^{-1}$ already led to long pauses in responding and omissions to respond. In pigs, doses equal to and exceeding 5 $mg.kg^{-1}$ tended to increase trial duration and the time taken to find the first food reward in pigs. The longer trial duration may have been caused by a longer time taken to visit the first hole and an increase of the number of erroneous hole visits before all the baits were found (RM errors).

Our findings suggest that conventional and minipigs differ in their sensitivity to the disruptive effects of biperiden with only the conventional pigs starting to refuse the higher doses. However, this apparent difference might have been caused by differences in body composition. Leanness significantly affects the volume of distribution of a drug and hence its adverse responses. Moreover, biperiden is highly lipophilic, rapidly entering the brain, but slowly entering fat tissue, from where it is slowly cleared (Yokogawa et al. 1990). Although we did not measure the proportion of body fat, we presumed that the conventional pigs were leaner than the minipigs. If this is the case, then minipigs are not necessarily less sensitive to the pharmacological effects of the biperiden, but instead have different pharmacokinetics. Although we found that biperiden did not impair retrieval of well-consolidated information from RM, and within-trial WM, it remains to be seen whether biperiden affects the learning process and/or memory consolidation. This can be assessed by administering biperiden before or immediately after the daily training trials during acquisition of the holeboard task. Biperiden has been found to impair consolidation and retrieval in a passive avoidance task with rats (Roldán et al. 1997, Kimura et al. 1999).

Our data confirm that biperiden is safe, even after administration of very high doses. The pigs were treated orally with up to 20 mg/kg^{-1} biperiden, a dose approximately 400-times higher than the therapeutic dose usually used in humans (see Fig. 1). We observed only mild non-cognitive adverse effects at the highest doses tested, such as mild signs of dry mouth (Gerretsen and Pollock. 2011), a typical side effect of anticholinergic drugs (de Leon. 2011). Liquid reinforcements could be used to get around the dry-mouth problem. It remains to be determined whether the reduced RM performance observed with the highest dose of biperiden really reflects cognitive deficits caused by a central action of biperiden or non-cognitive adverse

drug reactions. In the latter case, biperiden would not fulfill the requirements of a pharmacologically active cognition impairer in pigs (van der Staay et al. 2011). A potential limitation of the study is that none of the conventional pigs ate the mixture containing the highest dose of biperiden and one did not did not eat the mixture containing 15 $mg.kg^{-1}$ biperiden. This reluctance to eat is unlikely to have been due to a dry mouth, because this side effect developed about 30 minutes after drug ingestion. Dry mouth might have diminished the attractiveness or palatability of the M&M rewards. However, all Göttingen minipigs ate the food containing biperiden, even though they showed symptoms of dry mouth. The large volume of the mixture of crushed tablets, pig feed, and honey (the latter needed to stimulate pigs to eat the tablet mixture) may have delayed the absorption of biperiden, such that peak plasma levels, which were expected approximately 1–1.5 hours after drug administration on the basis of published pharmacokinetic data (Grimaldi et al. 1986), were reached later. This would have led to underestimation of the ability of biperiden to affect spatial memory. In the future, it might be more effective to administer pure biperiden, so that less "filler" is needed.

The present study did not provide evidence to support the conclusion drawn by Klinkenberg and Blokland (2011) that M1 receptor antagonists can be considered an alternative to scopolamine as cognition impairer, at least if conventional or minipigs are used as subjects. We suggest that biperiden should be evaluated further as putative cognition impairer in pigs, but using a different way to administer the drug. On the basis of our findings, a counterbalanced design could be used in a future study, e.g. involving the doses 1, 3, and 10 $mg.kg^{-1}$. The learning task could be made more difficult, and RM performance should be monitored daily, so that treatment can be started before performance reaches an asymptotic level, to prevent a ceiling effect. On the basis of our findings, minipigs can be used instead of conventional pigs or can serve as a translational model for other species. The effectiveness of biperiden treatment in conventional and minipigs clearly needs to be evaluated further before definitive conclusions can be drawn about the ability of biperiden to impair cognition in this species.

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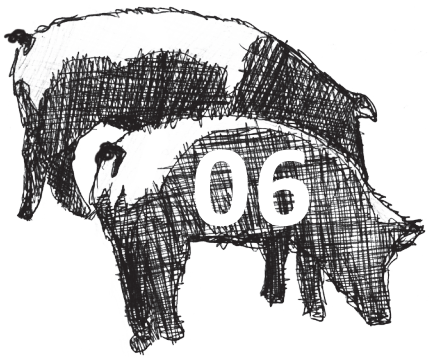

Chronic allopurinol treatment during the last trimester of pregnancy in sows: effects on low and normal-birth-weight offspring

Submitted manuscript

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Introduction

Infants born with a (very) low-birth-weight ((v)LBW) may have suffered from foetal growth restriction (Streimish et al. 2012), which refers to a pathological decrease in foetal growth rate (Burke et al. 2006). Low-birth-weight children are born with several risk factors for disease, morbidity and neonatal mortality (Gagnon. 2003). Risk factors increase if LBW is combined with being preterm (Baron et al. 2010, Bos et al. 2001). LBW after being carried to term gives better chances for survival, but the child is still at risk for developing several health as well as cognitive problems. (Bos et al. 2001, Chaudhari et al. 2004, Frisk et al. 2002, Ido et al. 1995, Kessenich. 2003, Martinussen et al. 2009, Silva et al. 2006, Yanney and Marlow. 2004) The possible causes for LBW are various and may be well-defined (i.e. chromosomal disorders, intra uterine viral infections) (Albertson-Wikland et al. 1993) or less clearly be attributable to causes such as smoking, obesity, air pollution or placental insufficiency (Biri et al. 2007, Gagnon. 2003). Placental insufficiency is seen as the most common cause (Sankaran and Kyle. 2009) and in general it can be said that a foetus suffering from placental insufficiency adapts to a lack of nutrients or oxygen (hypoxemia) by slowing down its growth rate (Barker. 1997, Gagnon. 2003). A foetus receiving inadequate oxygen and nutrient supply may protect its brain by a phenomenon called 'foetal brain sparing'. At the expense of blood trunk supply, more blood is diverted to the brain. (Barker. 2004, Cheema et al. 2009) However, this foetal adaptive reaction does not completely spare the brain and there might still be adverse consequences for later behaviour (Roza et al. 2008).

Cognitive deficits in humans associated with being born with (v)LBW are various and range from general learning problems (Frisk et al. 2002, O'Keeffe et al. 2003) to an increased risk for depression (Raikkonen et al. 2008), schizophrenia (Rifkin et al. 1994), anxiety, attention and hyperactivity disorders (Hayes and Sharif. 2009). Additionally, a reduced brain volume has been found in these children (Martinussen et al. 2009, Toft et al. 1995). (Also see Gieling et al. (2012))

As regards preventive therapies, except for optimizing time of delivery, treatments are not yet available (Sankaran and Kyle. 2009). Nowadays pregnant women in developed countries are monitored throughout their pregnancy and receive multiple heart rate and ultrasonographic (with additional Doppler) examinations. This will aid pre-partum recognition of the growth restricted foetus by ultrasound technicians and gynaecologists (Chaddha et al. 2004). Growth restriction by placental insufficiency can reliably be diagnosed during the second and third trimester of pregnancy and early treatment to limit the adverse consequences may immediately be started.

Brain damage and poor neurological outcome as a consequence of intra uterine growth restriction (IUGR) are less well understood. The mechanisms underlying altered neural development are yet unclear (Mallard et al. 2000, Yanney and Marlow. 2004). In case of compromised oxygen and nutrient supply to the brain, the foetus attempts to cope with the new situation but when compensatory mechanisms are insufficient, foetal distress may ensue and this can have far-reaching consequences extending into adult life. (Sankaran and Kyle. 2009) A consequence of oxygen

deprived brain tissue is neuronal cell damage or even cell death. This phenomenon has well been studied in many birth asphyxia studies (e.g. de Haan and Hasaart (1995) and Peeters and van Bel (2001)). Longer periods of mild oxygen deprivation are expected to occur in IUGR foetuses. These periods can be alternated with periods of re-oxygenation during which oxidative stress may occur and additional collateral damage is caused by free radicals produced (Biri et al. 2007, Peeters and van Bel. 2001, van Bel et al. 2006). Pharmacological intervention with neuroprotective substances, preventing the formation of, or scavenging the free-radicals produced, could possibly improve neurological outcome in these cases. When IUGR is detected during pregnancy, the mother could be treated until delivery with anti-oxidative drugs to prevent or limit the possible brain damage caused by free-radicals. One of these drugs is allopurinol (ALLO).

ALLO (1,5-dihydro- 4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one) has been found to reduce free-radical formation in for example pig, sheep and human foetuses (Boda et al. 1999, Masaoka et al. 2005, Peeters-Scholte et al. 2003). It is oxidised by the enzyme xanthine oxidase (XO) into the active metabolite oxypurinol (OXY) (Dallwig. 2010, Pacher et al. 2006, van Bel et al. 1998). The oxidising process inhibits the formation of damaging free radicals, and in higher concentrations, ALLO and OXY can also scavenge the free radicals present. (Moorhouse et al. 1987, Pacher et al. 2006) ALLO readily crosses the human and pig placenta and does not interfere with the parturition process if administered acutely during parturition (Boda et al. 1999). Torrance and colleagues (2009) suggested the therapeutic range for neuroprotection to be >2 mg.L-1 for ALLO and >4 $mg.L^{-1}$ for the active metabolite OXY. Therefore ALLO seems to be more potent compared to OXY. ALLO is currently applied in a clinical trial as a therapy preventing damage caused by acute birth asphyxia (Kaandorp et al. 2010b).

The neuroprotective capacities of ALLO were mainly studied in foetuses and neonates suffering from acute asphyxia during the parturition process. Treatment usually takes place during or after birth. Based on these studies it was suggested that treatment could have a positive effect if 1) ALLO was administered during or as early as possible after the occurring asphyxia and 2) the level of asphyxia was not too severe (i.e. that it did not induce irreversible damage) (Kaandorp et al. 2010b). Under these conditions it is more likely that ALLO treatment is beneficial for the cognitive outcome in children born with a LBW. Non invasive oral treatment could be started during pregnancy as soon as IUGR has been diagnosed and continue until delivery. The level of hypoxia is expected to be less severe in LBW children compared to children suffering from acute birth asphyxia.

The pig is increasingly used to study neurobehavioural dysfunction because of multiple advantageous characteristics such as its size and brain development (Gieling et al. 2011). Complementary to these advantages LBW piglets are a common phenomenon in commercial pig rearing (Gieling et al. 2012) due to increasing litter sizes and sow productivity (Beaulieu et al. 2010), but it is also seen in minipigs (Myrie et al. 2011). Therefore this species could potentially be used as a natural model for IUGR as mechanisms behind growth restriction are believed to be similar (Burke et al. 2006). Poor uteroplacental perfusion is seen as the main

cause of growth restriction in pigs (Blomberg et al. 2010) and occurs naturally in LBW newborn piglets from large litters (Bauer et al. 1998). Impaired cognitive performance related to LBW was shown in one of our earlier studies (Gieling et al. 2012). Therefore, this putative natural animal model was chosen to study the effects of ALLO.

As only little safety and efficacy data are available about the consequences of prolonged prenatal ALLO treatment in sows and their (IUGR) foetuses (Boda et al. 1999), we decided to address this question in piglets born with a low- and normal-birth-weight (NBW). Piglets were derived from sows treated with ALLO and from control sows. In a preliminary experiment we studied the pharmacokinetics of oral ALLO treatment in high pregnant sows to gain more insights in the plasma levels of ALLO and OXY in sows and piglets which could help with determining an appropriate dosing regimen for prolonged treatment. Subsequently, in an extensive study comprising a series of experiments, several birth and behavioural (emotion and cognition) measures were taken from piglets that had received prolonged ALLO treatment via the pregnant sows and from untreated controls. These measures included piglet characteristics at birth, piglet umbilical cord blood gas values, placental measures, behaviour in the open field and novel object test for emotionality, performance in a cognitive pig holeboard test for learning and memory and finally body, brain and spleen weight at slaughter (age range: 5 to 5.5 months).

We expected that chronic prenatal ALLO treatment would not interfere with the progression of pregnancy or parturition, development of the placenta or growth of the piglets. Further we hypothesised that no cognitive (brain and behaviour) or emotional measures of NBW piglets would be influenced by the treatment. LBW piglets treated with ALLO were expected to perform better in the cognitive holeboard test, and show less anxiety in the open field and novel object test compared to LBW controls. Brain, hippocampus and spleen weights were not expected to be influenced by ALLO treatment. Blood gas measures were taken to control for the occurrence of acute asphyxia as the piglets were all born naturally.

Material and Methods

Ethical note

The experiments were reviewed and approved by the local ethics committee at Utrecht University, and were conducted in accordance with the recommendations of the EU directive 86/609/EEC. All effort was taken to minimize the number of animals used and their suffering.

Pharmacokinetic studies

In experiment 1a, 1b and 2 pharmacokinetic data were collected in several pregnant sows treated with ALLO. A suitable dosing regimen was determined to be able to continue with experiment 3a and 3b. In experiment 3a and 3b (2 batches of animals) pregnant sows were treated with ALLO for a longer period during the third trimester of pregnancy or served as untreated controls. Details are described in the material and methods section per separate experiment.

Analysis of allopurinol in blood samples

In the experiments 1a, 1b and 2, bioanalysis of the blood samples to determine allo- and oxypurinol plasma levels was performed with reversed-phase high-performance liquid chromatography with UV-detection as described in (van Kesteren et al. 2006), except that the limit of detection was lower for this analysis (0.2 instead of 0.4 $\mu g.ml^{-1}$ for both ALLO and OXY) than in the study by van Kesteren.

Experiment 1a: pharmacokinetics of allopurinol in catheterised sows

Animals: Two pregnant sows [both a (Terra x Finnish landrace) x Duroc mix, 5th and 9th parity, see Table 1a] were used.

Housing: The sows were housed in separate pens but next to each other in an air-conditioned room with an average ambient temperature of 20° C immediately after permanent jugular catheterisation. Light was provided between 07:00h and 19:00h. The sows were fed twice daily with a standard pregnant sow diet (de Heus, Ede, the Netherlands), at 07:30h and 22.00h, supplemented with some silage. They always had *ad libitum* access to water. The floor of the pens was covered with sawdust and some enrichment material (ball or chewing sticks) was provided. Starting from the day of catheterisation surgery their health status and surgery wounds were monitored three times a day. The first ALLO administration was scheduled six days after surgery, on day 86 of pregnancy.

Treatment: Both sows were treated with ALLO orally (20 $mg.kg^{-1}$ b.w.) and via intravenous administration (20 $mg.kg^{-1}$ b.w.). Respectively, treatment days were day 99, 107 and 110 of pregnancy. For oral treatment ALLO tablets (100 mg, Ratiopharm, the Netherlands) were powdered and mixed with standard pregnant sow pellets. To prevent the sows from refusing the food based on an unfamiliar taste, they were habituated to eat these pellets mixed with some water and honey. The experimenter always observed the animal until the entire portion was consumed to confirm complete intake of the intended dose. Animals always consumed their food portion mixed with ALLO within 5-10 minutes.

PO administration of 20 $mg.kg^{-1}$ b.w. allopurinol (first measurement): On day 99 of pregnancy, both sows were treated orally with allopurinol (20 $mg.kg^{-1}$ b.w.). Blood samples were taken as described above. Fifteen minutes before administration a control sample of 4 ml blood was taken via the catheter and stored cold (4°C) in an EDTA tube. Starting 15 minutes after drug administration blood samples (4 ml) were collected every 15 minutes over a period of four hours (16 samples). Thereafter the sampling interval was increased to one hour over a period of seven hours (seven samples), followed by two additional sampling times at 20 and 25 hours p.a. respectively.

IV administration of 20 $mg.kg^{-1}$ b.w. allopurinol: On day 107 of pregnancy, both sows were treated with allopurinol (100 mg.ml-1 in 4 M NaOH solution, sterilized with a 0.2 µm filter, and administered within 24 h after preparation) administered through an intravenous drip. A control blood sample (4 ml) was taken just before the infusion started. The infusions lasted for approximately

20 minutes in sow 1, and 26 minutes in sow 2. After the allopurinol infusion the catheter was flushed with 40 ml of NaCl to prevent cross-contamination of the following blood samples. Blood samples were taken at 5, 15, 25, 35, 45, 55, 65, as well as after 125, 185 and 245 minutes (10 samples in total).

PO administration of 20 mg.kg^{-1} b.w. (second measurement): On day 110 of pregnancy, both sows were orally treated with allopurinol (20 mg.kg^{-1} b.w.). Fifteen minutes before administration a control blood sample (4 ml) was taken as described above. 225 Minutes after administration the sow was euthanized with pentobarbital (Euthanimal®, 150 mg.kg^{-1}, Alfasan, the Netherlands) administered through the catheter. Immediately after euthanasia, a Caesarean section was performed to deliver the piglets. Immediately after delivery, a blood sample was taken from every piglet by cardiac punction. All samples were drawn approximately 225 minutes after treating the sows with allopurinol, because the data from the first measurement showed that at this time point ALLO and OXY reached their peak concentration (C_{max}).

Experiment 1b: pharmacokinetics of allopurinol in sows and piglets

Animals: Two pregnant sows [both a (Terra x Finnish landrace) x Duroc mix, 5th and 6th parity, see Table 1a] were used.

Housing: The sows were group housed in a conventional housing system for pigs with an average ambient temperature of 20°C. Light was on between 07.00h – 22.00h and the sows were fed twice daily with a standard pregnant sow diet (de Heus, Ede, the Netherlands) at 08.00h and 16.00h in two separate pens. The sows had *ad libitum* access to water and their health status was monitored every day. The concrete floor was partially covered with sawdust and enrichment materials (ball and chewing sticks) were provided. Animals were given 5ml altrogenest (Regumate®, MSD Animal Health, the Netherlands) daily with their food starting four days prior to the expected farrowing date to prevent early farrowing.

Treatment: Oral ALLO (20 mg.kg^{-1} b.w.) administration was as described in experiment 1a. Starting on the afternoon before the day of drug administration, animals were fasted. Sows were ALLO treated twice (day 107 and 113 of pregnancy) with six days between treatments.

PO administration of 20 mg.kg-1 b.w.: On day 107 both sows were orally treated with allopurinol (20 mg.kg^{-1} b.w.) and one blood sample (4 ml) was taken from the external jugular vein 170 minutes after ALLO administration. ALLO and OXY plasma levels were determined to assure that these corresponded to the expectations based on experiment 1a. As this was the case, the experiment was continued on day 113 of pregnancy. Both sows were orally treated with allopurinol (20 mg.kg^{-1} b.w.). Fifteen minutes before administration a control blood sample (4 ml) was taken as described above. 170 minutes after administration the sow was euthanized with an i.v. injection with pentobarbital (Euthanimal®, 150 mg.kg^{-1}, Alfasan, the Netherlands). Immediately after euthanasia, a caesarean section was performed to deliver the piglets. Through a cardiac punction a blood sample was withdrawn from every piglet before the animal was euthanized.

The samples were drawn 170 minutes after ALLO treatment. This was done because the data gathered in exp. 1a showed OXY levels after ALLO administration to be very low in all piglets (and therefore therapeutically marginal). 170 Minutes was the average C_{max} of ALLO in the sows from experiment 1a and therefore at 170 minutes we expected to measure around the ALLO plasma level peak of the piglets too. Additionally three piglets were sampled at t=290 min.

Experiment 2: chronic allopurinol administration in pregnant sows I

Animals: Five pregnant sows [a (Terra x Finnish landrace) x Duroc mix, see Table 1a] were used.

Housing: The sows were housed in a group housing system for sows with automatic feeders, straw bedding and *ad libitum* access to water. The sows could freely enter an outside area where silage was provided. The ambient inside temperature ranged between 15 and 25°C and light was provided between 07:00 h and 22:00 h. Except for 1 kg of pellets mixed with the ALLO, the daily food ration (standard pregnant sow pellets, de Heus, Ede, the Netherlands) was distributed via an automatic feeder.

Treatment: Two sows were treated with allopurinol (15 $mg.kg^{-1}$ b.w.) for 30 days [± 2 days depending on the actual farrowing date, starting at day 86 (+ 1-3 days) of pregnancy] and three untreated sows served as controls (see Table 1a). ALLO tablets (300 mg, Ratiopharm, the Netherlands) were powdered and mixed with 1 kg of pellets, some honey and water. Animals were observed until all the food was consumed. Sows were weighed weekly to adapt the dose once a week corresponding to their weight gain or loss. As the Caesarean sections were planned on day 114 of pregnancy, all sows were given 5 ml altrogenest (Regumate®, MSD Animal Health, the Netherlands) daily with their morning food starting four days prior to the expected farrowing date to prevent early farrowing.

Procedures around delivery

Sows: Directly after restraining the sow, unconsciousness was reached by captive bold stunning. Piglets were delivered via a Caesarean section and the sow was bled. If a piglet was delivered the umbilical cord was tagged twice with coded umbilical clamps and cut in between the two clamps. This enabled linking a placenta to a specific piglet.

Piglets: Piglets were brought to a recovery room were they were dried and examined. Mucus was removed from the mouth and snout and the piglets were weighed, measured (length from nose to tail base and nose to end of the skull) and ear tagged. The piglets were housed in a small pen which was partially covered and heated with heat lamps and a heat mat (ambient temperature 32°C).

The average litter weight and the accompanying standard deviation (SD) were determined per litter. Piglets weighing the mean litter weight minus 1x the SD were classified as LBW. After excluding all LBW piglets from the litter a new litter mean was derived. Animals with a weight closest to this new mean and with the same sex as the LBW animal(s) from the litter were selected as NBW animals.

One to three LBW and one to three NBW animals were selected per litter, depending on availability. Directly after selecting animals from the litter, mixed blood was drawn from the umbilical cord and stored in EDTA tubes to determine allo- and oxypurinol levels.

The piglets were given sow colostrum via a bottle, syringe or tube (3 x 20 ml distributed over 3 hours). Colostrum from several other (untreated) sows was collected and frozen several weeks before the experiment. The colostrum from different sows was mixed and was slowly warmed up (30-40°C) in a lab water bath before feeding.

Placental measures: Piglet-matched placentas were derived after the caesarean section. Two marked umbilical cord clamps were attached to the umbilical cord and the cord was cut in between. When the placenta could be removed from the sow it was stored (4°C) and examined within one week to look at any possible teratogenic effects of ALLO treatment on placental development. Measures included: placenta length (measured along the inside of the placenta from one end to the other); placenta width (measured along the base of the placenta at the broadest point); and placenta circumference (measured by placing a piece of rope exactly around the placenta). All placentas were weighed (10 g accuracy, Breuer Weegtechniek JB-800, Boxtel, the Netherlands). Scaled pictures were taken from above to calculate surface area with PDF-Xchange Viewer 2.5. Only placentas that had a traceable tag, were mainly undamaged and could be unfolded were measured. Numbers per sow are shown in Table 3.

Experiment 3a and 3b: chronic allopurinol administration in pregnant sows II

Animals: Twelve pregnant sows [(Terra x Finnish landrace) x Duroc mix divided over two batches of six sows, with two months between batches, see Table 1b] were used.

Housing: Until one week before farrowing, the sows were housed in a group housing system for sows with automatic feeders, straw bedding and *ad libitum* access to water. The sows had free access to an outside area where silage was provided. The ambient inside temperature ranged between 15 and 25°C and light was provided between 07:00 h and 22:00 h. Except for one kg of pellets mixed with ALLO, the daily food ration (standard pregnant sow pellets, de Heus, Ede, the Netherlands) was distributed via an automatic feeder.

Treatment: From twelve pregnant sows (see Table 1b), six were treated with ALLO (15 $mg.kg^{-1}$) for 30 days (± 2 days depending on the actual farrowing date, starting at day 86 (+ 1-3 days) of pregnancy) as explained in experiment 2. Six untreated sows were used as controls. The last ALLO dose was administered on the day of farrowing. The dose of 15 $mg.kg^{-1}$ b.w. was based on a simulation of the plasma-concentration time curve established from two individual sub-experiments in two sows (described in exp. 1a).

Procedures around delivery

Sows: One week prior to the expected farrowing date all sows were moved to a conventional farrowing stall (ambient temperature 20-23°C, with floor heating, 30°C, in the piglet area) and were housed there till the piglets were weaned. Food was provided automatically two times a day and access to water was *ad libitum*.

Piglets: After taking all birth measures, piglets returned to the sow directly to drink colostrum. Starting at 2-3 days of age extra artificial milk for piglets (Milkiwean, Trouw Nutrition, the Netherlands) was provided in the pen via a drinking bowl. At 3 days of age all piglets were preventively given an iron injection. When birth diarrhoea occurred, all piglets from the affected litter were treated orally with colistine (Enterogel, Virbac Animal Health, Barneveld, The Netherlands) for 3-5 days. Crippled piglets before or after weaning where treated with ampicillin (Ampicillan 20%, Alfasan Nederland B.V., Woerden, The Netherlands) for 3-5 days and if necessary an analgesic with meloxicam was administered once (Novem 20 $mg.ml^{-1}$, Boehringer Ingelheim Vetmedica GmbH, Ingelheim, Germany). No tail docking, castration or other invasive mutilatory procedures were applied in the selected LBW and NBW piglets.

After the piglets had reached 3.5-4 weeks of age the sow was removed and the piglets were weaned. After 1-1.5 additional week(s) in the farrowing pen the selected LBW and NBW piglets were mixed and moved to the two pens in the experimental unit.

The experimental pens (3x5 m) had a concrete floor. A piglet nest (3x1 m) could be accessed through rubber flaps. The nest floor was covered with rubber mats, a heat mat (20-30°C 70x40 cm), saw dust and straw. The pen floor was also covered with straw. Food was provided *ad libitum* during the first 1.5 weeks and twice a day during the rest of the experiment (⅓ before and ⅔ after testing) and was scattered on the pen floor. Water was always available trough an automatic drinker. Extra enrichment materials were provided (balls, chewing sticks).

Birth measures: Sows were observed constantly starting three days prior to their expected farrowing date. With the onset of labour, at least two experimenters were present to catch the piglet when it was expelled and code the umbilical cord with surgical silk tagged with 'knots'. These knots were linked to the piglets' ear tag, given after blood sampling. The placenta side of the umbilical cord was tagged and the other side was clamped with a kocher. The cord was cut in between at least 7-15 cm away from the piglet. Slightly above the kocher the umbilical cord was cut again and some mixed blood (max. 1 ml) was gathered in a 1.5 ml Eppendorf tube. Directly after blood sampling the umbilical cord was disinfected with Betadine and clamped and/or sutured till the bleeding stopped. The blood was directly drawn from the tube into a labelled 1 cc syringe (w/-25 Balanced Heparin, Luer Tip Cap, Westmed, USA) and put on ice. Within 20 minutes the blood sample was cleared from air and analysed with a portable blood gas analyser (ABL80 SC80, Radiometer, Zoetermeer, the Netherlands) with a sensor cassette (100/30 Full, no Glu, QC[3]) and pH, $_pCO_2$, pO_2, $_cNa+$, $_cK^+$, $_cCa^{2+}$, $_cCL^-$ and Hct were measured.

Table 1a. Overview of the sows and piglets used in experiment 1a, 1b and 2.
All animals are a (Terra x Finnish landrace) x Duroc mix.

Animals	Group	Parity			
Exp. 1a			**Weight (kg) at start**		**Allopurinol treatment**
Sow 1	n/a	5	254		20 mg.kg^{-1} PO, 20 mg.kg^{-1} IV
Sow 2	n/a	9	292		20 mg.kg^{-1} PO, 20 mg.kg^{-1} IV
Exp. 1b					
Sow 3	n/a	6	315		20 mg.kg^{-1} PO
Sow 4	n/a	5	273		20 mg.kg^{-1} PO
Exp. 2			**Litter size**	**Av. lit. weight (g)**	
Sow 1	Allo	9	13	1720.83	15 mg.kg^{-1} PO (repeated daily)
Sow 2	Allo	7	15	1617.47	15 mg.kg^{-1} PO (repeated daily)
Sow 3	Control	7	15	1308.33	n/a
Sow 4	Control	7	14	1040.00	n/a
Sow 5	Control	6	16	1000.00	n/a

Table 1b. Overview of the sows and piglets used in experiment 3a and 3b. All animals are a (Terra x Finnish landrace) x Duroc mix. Only piglets from exp. 3a and 3b (batch I and II) were tested behaviourally. ALLO sows were treated with 15 mg allopurinol per kg b.w. once daily.

Animal	Group	Parity	Litter size	Av. lit. weight (g)	♂ / ♀	n	LBW and NBW piglets selected for behavioural testing					
Exp. 3a						Plac[1]	Gas[2]	LBW (gender- birthweight)			NBW (gender- birthweight)		
Sow 1	Allo	7	14 (+ 2†)	1303.21	10 / 4	12	2	♂ 755g	♂1040g		♂ 1300g	♂ 1375g	
Sow 2	Allo	4	13 (+ 4†)	1276.54	7 / 6	12	3	♀ 890g	♂ 980g		♀ 1400g	♀ 1480g	
Sow 3	Allo	2	12 (+ 2†)	1275.83	5 / 7	13	2	♂ 875g			♂ 1410g		
Sow 4	Control	6	18 (+ 1†)	1362.67	11 / 7	7	4	♂ 715g	♂ 720g	♀ 1080g	♂ 1660g	♀ 1710g	♂ 1750g
Sow 5	Control	4	17	1338.82	13 / 4	6	2	♂ 930	♂ 1070g	♂ 1085g	♂ 1370	♂ 1440g	♂ 1340g
Sow 6 *	Control	3	10	1855.50	7 / 3	10	2	-	-	-	-	-	-
Exp. 3b						Plac[1]	Gas[2]	LBW (gender/ birthweight)			NBW (gender/ birthweight)		
Sow 7	Allo	9	9 (+ 4†)	1607.79	5 / 4	4	5	♂ 1155			♂ 1740		
Sow 8	Allo	2	18	1439.44	11 / 7	15	10	♀ 860	♂ 1115		♀ 1525	♂ 1700	
Sow 9	Allo	2	12	1546.67	6 / 6	12	4	♀ 1040	♀ 825		♀ 1665	♀ 1590	
Sow 10	Control	8	8	1304.38	7 / 1	5	4	♂ 870			♂ 1415	♂ 1460	
Sow 11 *	Control	2	5	2028.00	2 / 3	5	2	-	-	-	-	-	-
Sow 12	Control	10	19	0858.89	12 / 7	7	5	♂ 470			♂ 950		

*No piglets from these litters were selected for the open field and holeboard test as no proper LBW animals were present in those litters. 1 number of piglets for placental measures, 2 number of piglets for blood gas measures.

After measuring and weighing (see experiment 1 for procedures) the piglet returned to the sow and stayed here until weaning. LBW and NBW piglets were determined per litter according to the criteria described in 'experiment 2'.

Placental measures: Piglet-matched placentas were derived and stored (4°C) until examination within one week to look at any possible teratogenic effects of ALLO treatment on placental development. Placenta measures taken are the same as described in experiment 2.

Behavioural testing

Open field and novel object test: During the 5th week after birth, 1 week after moving and mixing, an open field and novel object test was performed once with every animal. In a random order but per pen an animal was separated and let into a corridor leading to the test arena. It was let into a small waiting box covered with saw dust, as on the test floor. After approximately 30 seconds the animal was let into the open field arena and the door was closed. The arena (250 x 205 cm (first batch) and 250 x 150 cm (second batch)) was fenced with wooden and synthetic partitions, at least 1.2 m high. A radio was playing on the background to mask environmental noise. The area was filmed from above and a sheet placed in front of the monitor divided it in 16 partitions. Behaviour could be observed from the screen and scored with the custom made software Observe6. Vocalizations were scored by a third observer, not visible by the pig.

The total duration of the test was ten minutes. After five minutes an unknown object (a colourful plastic tambourine) was suddenly lowered by a rope in the middle of the arena and made an unexpected noise when touching the floor. Two additional behaviours were scored from this moment on: 'touching the object' and 'looking at the object'. After ten minutes the animal was led back to its pen.

Holeboard testing: The cognitive pig holeboard apparatus (manufacturer Ossendrijver BV, Achterveld, the Netherlands) consisted of a square arena (530 x 530 cm) surrounded by a narrow corridor (width 40 cm). Via this corridor, four guillotine entry doors could be reached to access the arena. In the arena a 4 x 4 matrix of food bowls was placed. Rewards in a bowl could be found by lifting the balls on top with the snout. For a detailed description of the apparatus see Gieling et al. (2012). The holeboard apparatus could be accessed via a group waiting pen. Animals were always tested individually in the apparatus and a radio played continuously to mask sudden background noises.

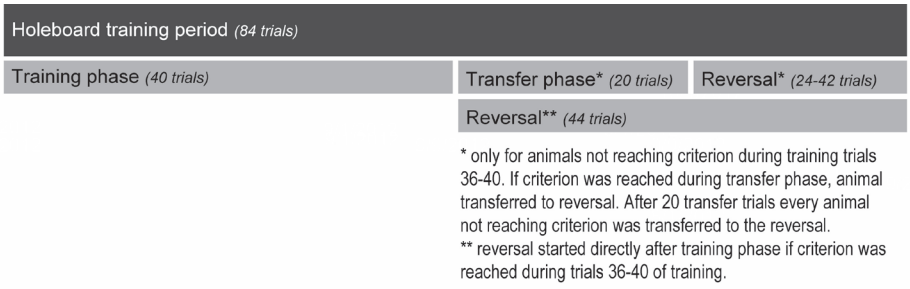
Each food bowl was equipped with a magnet sensor and every ball with a magnet. Ball-lifts were registered through an interface (LabJack Data Acquisition Device, LabJack Corporation, USA). The interface was connected to a laptop with custom made software to control the experiment and process and register the data (Exp. Control for Utrecht University, Blinq Systems, Delft, the Netherlands). All measurements were recorded real time and automatically. A visit was counted when a pig lifted the ball on top of the food bowl with its snout. A signal between a magnet sensor placed under the false bottom and a magnet in the ball was now disconnected. Via an interface (LabJack) a signal was sent to the software

mentioned previously. Output was stored in Microsoft Excel format (calculated data) and as raw data in a text file.

Habituation started five weeks after birth. An experimenter sat in the pen and touched the animals gently when possible. M&M chocolates and some corn cob mix were given to attract their attention and get them used to the rewards. Piglets were habituated to the corridor leading to the holeboard waiting area and the waiting area itself (a pen with an automatic drinker and straw bedding). As a group they were led in to the holeboard using different entry doors. All food bowls were rewarded with M&M chocolates. After three group sessions the group was split in two and habituation to the holeboard was repeated, using only M&M's as rewards. Finally the animals were tested in groups of two individuals (approx. 4 times) and alone (approx. 4 times). The entire habituation period lasted 13 working days.

An animal was defined ready for testing when it was able to stay in the holeboard for at least 60 seconds while searching for rewards under the balls. For the first training trial, all animals entered the holeboard through door nr. 1. On all following trials, the entry door was assigned randomly by the software. A specific door was never assigned to an animal more then twice in a row. Every trial lasted till the 4th reward was found or ten minutes had elapsed, whichever event occurred first. Every animal was assigned two successive trials a day (one session) in a random order. The training phase in which every animal was assigned one specific configuration of rewarded bowls (4 out of 16) lasted for at least 40 trials per animal. Four different configurations were used (the configuration as depicted in Fig. 4E in van der Staay et al. (2012) and three variants twisted 90°, 180° and 270°). A performance criterion (session average reference memory performance > 0.7 for at least two consecutive sessions) was set before an animal was allowed to switch from the training configuration (training phase) to a new one (reversal). All animals switched to the reversal if not reaching criterion after a maximum number of 60 training trials (transfer phase). In total all animals were trained for an equal number of trials (84), although the number of training and reversal trials differed per animal (outlined in Fig. 1).

Fig. 1. Timeline holeboard training



Holeboard measures are memory, motivation or strategy related. (Re)visits to rewarded bowls, (re)visits to unrewarded bowls and total trial duration were measured. For scoring revisits two specific rules were applied: a revisit only was

counted as such if at least ten seconds had elapsed between the previous visit to the same bowl or when another bowl was visited in between.

Several measures were derived from the raw data:

Working memory ratio (WM): (number of rewarded visits) / (number of visits and revisits to the rewarded set of bowls). WM is seen as a short term memory measure, reflecting the ability of the animals to avoid revisiting baited bowls (Arts et al. 2009).

Reference memory ratio (RM): (number of visits and revisits to the rewarded set of bowls) / (number of visits and revisits to all bowls). RM is seen as a long term memory measure and is an index for the ability of an animal to discriminate between baited and unbaited holes (Arts et al. 2009).

Ratio measures were used as these are less biased by incomplete trials, in which the animal does not collect all rewards (van der Staay et al. 2011).

Trial duration (TD): the time elapsed between entering the Holeboard and finding the last reward with a maximum of 600 seconds if not all rewards were found.

Inter visit interval (IVI): the average time between visits to bowls (s).

Trials to criterion (TC): The number of trials an animal needs (with 40 as a minimum) till reaching criterion to start with reversal training.

Response flexibility (RF): Performance (WM, RM, TD and IVI) of the last trial block of the first configuration is compared with the performance of the first trial block of the second configuration (reversal). The larger the difference (delta), the more difficulty an animal showed to adapt to a new situation.

Choice Correspondence (CC): Visiting order of first visits to the four rewarded bowls. This measure can give insight in the strategy an animal applies to solve the task. An animal could repeatedly follow the same strategy or alter it depending on the situation (e.g. entry door) to maximize gain. (van der Staay et al. 2012) CC is calculated according to the rules described in (van der Staay et al. (2012), Fig. 2. With no strategy animals would score an average of 1.72 and a higher performance indicates use of a (partial) strategy. When the exact same visiting order would be applied repeatedly, the maximum score of 4 would be reached. Additionally, deltas of mean CC scores per door per BW group minus 1.72 were calculated to analyse whether the scores significantly differ from zero, i.e. whether the animals employ a strategy to find their rewards.

Errors per reward (EpR): Shows the number of errors (incorrect (re)visits) an animal makes before finding reward 1-4. The errors are counted per reward found (before finding reward 1, between finding reward 1 and 2, 2 and 3 and 3 and 4) and not accumulated over rewards. Every trial results in four numbers, displayed in a fixed order. These four numbers are compared with each other per block of trials

(four trials per block) and per BW group to analyse whether the EpR stays level at a specific moment in training or if it increases after finding 1, 2 or 3 rewards. This measure can be used in a descriptive way to interpret the difficulty level of the test per treatment group. If the number of errors increases when later rewards are still to be found, probably memory load is increasing or executive-attention to fulfil the task correctly is declining or eventually lost. An executive-attention component deficit is suggested to be one of the hypotheses behind cognitive impairment in IUGR children (Geva et al. 2006).

Data analyses

Pharmacokinetics of allopurinol: Plasma ALLO and OXY analysis in the sows after PO and IV administration was performed in a non-compartmental model. Foetal plasma concentrations were not analysed statistically because they were measured at only one time-point after administration.

Placental measures: The large number of untraceable placentas precluded an analysis of the effects of birth weight. Consequently, this factor was not included in the analysis of the placenta measures. Using SPSS 16.0 for Windows placenta measures (exp. 2, 3a and 3b) were analysed using a linear mixed model with 'treatment' as a fixed and 'sow' as a random factor. 'Litter size' was used as covariate as the litter size is a major determinant of the weight of the piglet in a litter, with larger litters having smaller piglets (van der Lende and de Jager. 1991).

Pearson correlation coefficients and Spearman's correlation coefficients were calculated to check for correlations between placenta variables. Piglet birth weight is included in the analyses to search for correlations between birth weight and placental variables. Data were analysed with SPSS 16.0 and SAS 9.2.

Piglet measures and blood gas data: Using SPSS 16.0 for Windows, piglet measures and blood gas data were analysed using a linear mixed model for the umbilical cord blood gas values and piglet measures data. 'Treatment' was set as a fixed factor and 'sow' as a random factor. 'Litter size' was used as covariate.

Open field and novel object data: Open field measures include line crossings (LC, an activity measure), the number of vocalizations (V), the number of defecations (D) (all during ten minutes) looking at the novel object (LNO) and touching the novel object (TNO) (both 5 min observations).

Using SPSS 16.0 for Windows, the data were analysed using a linear mixed model with 'treatment' as a fixed factor, 'sow' as a random factor and 'litter size' as a covariate. Birth weight class (LBW or NBW) was added as well as the interaction 'treatment*birth weight class' as fixed factors. Each variable was checked for normality by plotting parameter estimates against parameter residuals in a Q-Q and scatter plot. Significance level was fixed at ≤ 0.05.

Holeboard data: The animals were trained for two consecutive trials a day (one session). For each measure, block mean values of four trials (two sessions) were calculated (methods adapted from Arts et al. (2009) and Gieling et al. (2012)).

The data was analyzed with SAS 9.2. NBW and LBW piglet data was averaged per sow (treated or control) and the repeated measures data (blocks of four trials each, or doors) of each sow were used in the analysis. Therefore, for each variable two measures per trial block for each sow were tested in a General Linear Model for Repeated Measures with trial blocks or doors as second repeated measures factor. Every variable was checked for normality with a Shapiro-Wilk test for normality. Significance level was fixed at ≤ 0.05.

Body, brain and spleen weights: The data was analyzed with SAS 9.2. NBW and LBW piglet data was averaged per sow (treated or control). To calculate relative weights, the weight of the brain or spleen was divided by the final body weight of the animal. The two variables for each sow were tested in a General Linear Model for Repeated Measures with birth weight as repeated measures factor. Every variable was checked for normality with a Shapiro-Wilk test for normality. Significance level was fixed at ≤ 0.05.

Results

Pharmacokinetics of IV and PO allopurinol treatment

Experiment 1a: pharmacokinetics in catheterised sows

Sows: A non-compartmental analysis was performed on the data of the (IV) infusion of an ALLO dose of 20 $mg.kg^{-1}$ b.w. and a PO administration of 20 $mg.kg^{-1}$ b.w. The results are summarised in Table 2. In the two sows the mean C_{max} for respectively PO and IV dosing of ALLO 20 $mg.kg^{-1}$ was 6.49 and 23.81 $\mu g.ml^{-1}$ for ALLO and 4.84 and 7.41 $\mu g.ml^{-1}$ for OXY. After oral dosing ALLO and OXY plasma levels were below the limit of detection (LOD) at >25h (ALLO) and >25h (OXY) for sow 1, and 25h (ALLO) and >25h (OXY) for sow 2. After IV dosing, in both sows plasma ALLO and OXY levels were still detectable >4 hours after administration.

Piglets: A complete set of plasma concentrations is only available from the piglets of sow 1, from which all 17 piglets were sampled. The average ALLO and OXY plasma levels were 4.10 $\mu g.ml^{-1}$ and 0.45 $\mu g.ml^{-1}$ respectively, 225 minutes after oral administration of ALLO to their mother (Fig. 2, panel B). The sow plasma level samples at 225 min were 7.69 $\mu g.ml^{-1}$ (ALLO) and 5.56 $\mu g.ml^{-1}$ (OXY).

Table 2. Pharmacokinetic parameters of plasma allopurinol and oxypurinol in sows, non-compartmental analysis.

Parameters	Units	Dose and route of administration			
		20 mg.kg^{-1} PO (ALLO)		***20 mg.kg^{-1} IV (ALLO)***	
		Sow 1	*Sow 2*	*Sow 1*	*Sow 2*
		Mean	*Mean*	*Mean*	*Mean*
C_{max}	µg.ml^{-1}	6.00	6.98	19.85	27.77
T_{max}	hr	4.00	2.75	0.60	0.52
λ z	1.hr^{-1}	0.46	2.60	0.65	0.70
HL λ z	hr	1.50	0.37	1.07	0.99
AUC_{last}	hr.µg.ml^{-1}	22.83	21.46	29.72	35.07
$AUMC_{last}$	hr.hr.µg.ml^{-1}	72.43	62.80	43.38	48.65
MRT_{INF}	hr	3.20	2.93	1.57	1.60
		20 mg.kg^{-1} PO (OXY)		***20 mg.kg^{-1} IV (OXY)***	
C_{max}	µg.ml^{-1}	4.51	5.17	6.64	8.17
T_{max}	hr	4.50	4.00	0.60	0.52
λ z	1.hr^{-1}	2.67	1.21	0.29	0.28
HL λ z	hr	0.26	0.57	2.37	2.43
AUC_{last}	hr.µg.ml^{-1}	17.50	20.22	17.41	22.25
$AUMC_{last}$	hr.hr.µg	63l31	71.22	32.93	43.56
MRT_{INF}	hr	3.66	3.61	3.65	3.77
AUC_{oxy}/AUC_{allo}		0.77	0.94	0.59	0.63

C_{max} = maximal plasma concentration; T_{max} = time to reach C_{max}; λ z = elimination constant; HL λ z = elimination half-life; AUC_{last} = Area Under the plasma concentration-time Curve from time zero to time of last measurable concentration; $AUCM_{last}$ = Area under the first moment of the plasma concentration-time curve from time zero to time of last measurable concentration; MRT_{INF} = Mean Residence Time to infinity; AUC_{oxy}/AUC_{allo} = Area Under the Plasma concentration time Curve oxypurinol / Area Under the Plasma concentration time Curve allopurinol. PO at 99 and IV measurements at 107 days of gestation (first measurements of exp. 1a)

Experiment 1b: pharmacokinetics in sows and piglets

As shown in Fig. 2, panel C and D, five piglets from sow 3 and six piglets from sow 4 were sampled at 170 minutes after ALLO administration. Average ALLO plasma levels were found to be 1.80 µg.ml^{-1} and 1.49 µg.ml^{-1} respectively. Average OXY plasma levels of 0.53 µg.ml^{-1} (sow 3) and 0.42 µg.ml^{-1} (sow 4) were found. The sows were sampled at the same time point as their piglets. The results of this experiment were also used to establish the dosing regimen for the long-term application of ALLO to pregnant sows. Considering a desirable plasma concentration in the piglets of at least 2 µg.ml^{-1} ALLO, a simulation of the kinetic parameters obtained from sow 1 and her piglets suggested that a minimum oral dose of 14 mg.kg^{-1} b.w. would be required to achieve steady state conditions in piglets and to exclude drug accumulation in the parent animal. Hence it was decided to apply an oral dose of 15 mg.kg^{-1} b.w. in the experiments 2 and 3.

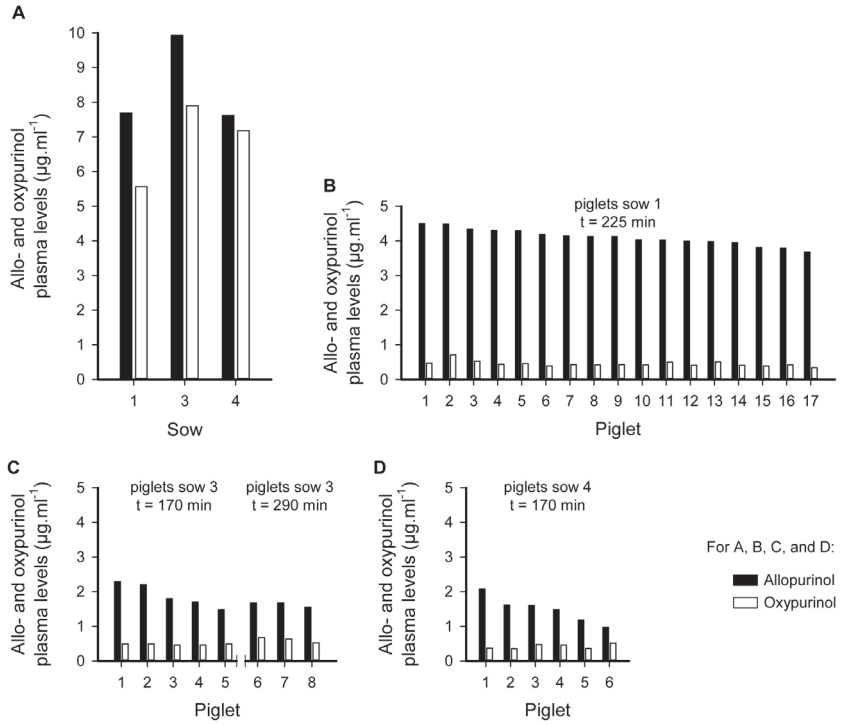


Fig. 2. Allo- and oxypurinol plasma levels (µg.ml^{-1}) measured in 3 different sows **(panel A)** at t = 170 min (sow 3 and 4, exp. 1b) and t = 225 min (sow 1*, exp. 1a) after oral administration of allopurinol (20 mg.kg^{-1}) and plasma levels of their piglets at t = 170 min (exp. 1b, **panel C and D**), t = 225 (exp. 1a, **panel B**) and t = 290 min (exp. 1a, **panel C**) after delivery by caesarean section.
*Note: sow 2 excluded due to unreliable plasma levels in both sow and piglets

Placental measures (exp. 2, 3a and 3b)

A total of 125 placentas derived from 17 sows were collected (Table 3) during three data collection periods (one Caesarean section (exp. 2), two natural deliveries (exp. 3a/b)). From these 125 placentas, 57 could be linked to specific piglets. From sow 12 (see Table 1), no piglets could be linked to their placentas.

Placenta length was found to be shorter in ALLO treated piglets compared to control piglets. No other effects of treatment were found for placenta width, circumference and surface area (see Table 3).

Placenta length was found to be correlated with placenta circumference (r = 0.945, P = 0.000), placenta surface area (r = 0.597, P = 0.015) and placenta weight (r = 0.502, P = 0.040). Placenta width was only correlated with placenta weight (r = -0.614, P = 0.009). Placenta circumference correlated with placental length (r = 0.945, P = 0.00), placenta surface area (r = 0.543, P = 0.024) and piglet birth weight (r = 0.642, P = 0.007). Placenta surface area was found to be correlated with placenta length (r = 0.597, P = 0.015) and placenta weight (r = 0.700, P = 0.002). Placenta weight correlated with placental length

(r = 0.502, P = 0.040), placenta width (r = -0.614, P = 0.009) and placenta surface area (r = 0.700, P = 0.002). Finally, piglet birth weight correlated with placenta circumference (r = 0.642, P = 0.007) but not with any of the other measures. All correlations are shown in Table 3.

Table 3. Correlation diagram. Correlations (Pearson's correlation coefficient) between variables are shown.

		Placenta length	Placenta width	Placenta circumference	Placenta surface area	Placenta weight	Piglet birth weight
Placenta length	Correlation	1	-0.176	0.945	0.579	0.502	0.488
	Sig. (2-tailed)		0.500	0.000	0.015	0.040	0.055
	N	17	17	17	17	17	16
Placenta width	Correlation		1	-0.027	-0.270	-0.614	0.188
	Sig. (2-tailed)			0.917	0.295	0.009	0.486
	N		17	17	17	17	16
Placenta circumference	Correlation			1	0.543	0.361	0.642
	Sig. (2-tailed)				0.024	0.154	0.007
	N			17	17	17	16
Placenta surface area	Correlation				1	0.700	0.180
	Sig. (2-tailed)					0.002	0.504
	N				17	17	16
Placenta weight	Correlation					1	-0.045
	Sig. (2-tailed)						0.867
	N					17	16
Piglet birth weight	Correlation						1
	Sig. (2-tailed)						
	N						16

Piglet birth measures (exp. 2 (placentas only), 3a and 3b)

Full body length was found to be longer in allopurinol treated piglets compared to control piglets. No treatment effects were found for snout length, birth weight and ponderal index (Table 4).

Piglets finally selected as LBW and NBW animals for behavioural testing ranged at birth between 470 g and 1155 g (LBW: ALLO average weight 956 g; CONT average weight 867 g) and 950 g and 1750 g (NBW: ALLO average weight 1519 g; CONT average weight 1455 g).

Blood gas measures (exp 3a and 3b)

A maximum of 2-10 umbilical cord mixed blood samples could be collected from piglets per sow as it was not possible to draw blood from all umbilical cord veins after the cord was cut or broken. The pH values of all sampled piglets did not include any values below 7.0. The pCO2 values measured all stayed below 100 mm Hg, although two samples did have a very low pCO2 concentration (< 25 mm Hg). No effect of allopurinol treatment was found on any of the blood gas parameters. The data is shown in Table 4.

Table 4. Piglet birth measures.

Measure										
Placenta	*MEAN (ALLO)*	*SEM (ALLO)*	*N (ALLO)*	*MEAN (CONT)*	*SEM (CONT)*	*N (CONT)*	*F*	*DF*	*denominator DF*	*P <*
Length (cm)	63.86	1.64	74	74.95	2.60	51	**4.886**	1	14.335	**0.044**
Width (cm)	15.67	0.26	70	15.96	0.28	48	0.249	1	13.398	0.626
Circumference (cm)	147.47	3.47	74	167.25	5.52	51	2.557	1	15.216	0.130
Surface area (cm^2)	583.64	30.73	69	624.97	37.32	45	0.320	1	13.888	0.581
Weight (g)	0.273	0.01	74	0.266	0.01	51	0.200	1	0.010	0.663
Piglets	*MEAN (ALLO)*	*SEM (ALLO)*	*N (ALLO)*	*MEAN (CONT)*	*SEM (CONT)*	*N (CONT)*	*F*	*DF*	*denominator DF*	*P <*
Birth weight (g)	1470.97	31.61	105	1255.08	37.55	103	3.044	1	12.463	0.106
Full length (cm)	37.296	0.34	93	33.203	0.49	72	**6.347**	1	12.421	**0.026**
Snout length (cm)	12.350	0.18	94	11.232	0.18	74	0.081	1	12.687	0.387
Ponderal index	27.693	0.46	93	33.502	1.39	72	1.479	1	11.907	0.247
Blood gases	*MEAN (ALLO)*	*SEM (ALLO)*	*N (ALLO)*	*MEAN (CONT)*	*SEM (CONT)*	*N (CONT)*	*F*	*DF*	*denominator DF*	*P <*
pH	7.377	0.02	26	7.420	0.02	19	1.603	1	8.686	0.238
pCO2	47.35	1.80	26	45.58	2.15	19	0.008	1	8.229	0.930
pO2	39.62	6.50	26	52.37	8.98	19	1.217	1	3.540	0.339
Hct	25.56	0.65	26	26.67	1.86	19	0.315	1	8.910	0.588

Differences in birth measures between piglets from sows treated with allopurinol (n=6) and controls (n=4). Full body length (cm) = snout – tail base, snout length (cm) = snout – end of skull, birth weight (g) and ponderal index = weight/$length^3$.

Open field and novel object (exp 3a and 3b)

In total 37 piglets were tested in the open field test with the number of piglets tested per sow ranging between 1-3 per birth weight group. The only effect found was that LBW piglets vocalised more than NBW animals ($F_{1,7}$=4.895, p=0.036; mean LBW 327.61 (SEM 56.01), mean NBW 238.05 (SEM 54.61)). No effects of treatment or treatment*birth weight class were found.

Holeboard

TD, WM, RM, IVI, RF, and TC: Holeboard results are depicted in Table 5 and Fig. 3 and 4 Training improved performance (block effect for TD, WM, RM and IVI both for the training and the reversal phase). A blocks*treatment interaction effect was found for the measure IVI during the reversal phase ($F_{5,40}$ = 3.28, P < 0.0141). ALLO treated piglets had a longer IVI during block 13 and 14 of reversal training (contrast variables block 13: $F_{1,8}$ = 5.64, P < 0.0001; block 14: $F_{1,8}$ = 8.28, P < 0.0206). No other treatment, birth weight or treatment*birth weight group effect was found for the training or reversal phase for the measures TD, WM, RM and IVI. Comparing the RF and TC of the four groups, no differences were found.

Choice Correspondence: Comparing the visiting order of first visits to rewarded bowls per block of four trials for the first ten blocks of training, none of the four

groups significantly changed their strategy over the blocks ($F_{9,63}$=0.56, p=0.8211) and the slopes showed no differences between groups. The average CC calculated per door over 40 training trials didn't show different levels food searching strategies per door, neither did treatment or BW group influence CC. Delta's of mean CC score per door per BW group minus performance at chance level (1.72) show that strategy performance for all doors was significantly above random performance level (LBW group: door 1 t_9= 2.86, p=**0.0.0187**, door 2 t_9= 5.03, p= **0.0007**, door 3 t_9=3.74, p=**0.0046**, door 4 t_9=4.37, p=**0.0018**; NBW group: door 1 t_9=3.24, p=**0.0101**, door 2 t_9=4.64, p=**0.0012**, door 3 t_9=4.18, p=**0.0024**, door 4 t_9=4.08, p=**0.0027**).

Errors per reward (EpR): During the first and the 10th block of training, the number of errors made before finding the first rewarded bowl and between finding the following rewards increased in order of the rewards obtained (see Fig. 4. block 1: $F_{1,7}$=12.53, p<0.0001; block 10: $F_{3,24}$=6.79, <0.0018). ALLO treatment or birth weight or their interaction didn't affect EpR.

Brain, hippocampal, and spleen weight

No overall influence of ALLO treatment on final body weight of the pigs (age between 5-5.5 months) was found, but there was a marginal interaction between birth weight and treatment. LBW control animals seemed to have a lower body weight compared to the LBW animals treated with ALLO. This didn't seem to be the case for the NBW groups. LBW animals had a lower end (slaughter) weight compared to NBW animals ($F_{1,8}$ = 5.20, p < 0.0047). Brain and hippocampus weights did not differ between the ALLO treated animals and the controls either. Spleen weight, however, was marginally lower in the ALLO treated animals ($F_{1,8}$ = 4.23, p <0.0073, see Fig. 5D). Brain and hippocampus ratio measures were influenced by birth weight but not by treatment (Table 6). In absolute terms LBW brains weighed significantly less compared to NBW brains (analysis not shown, mean brain weight LBW animals 100.5 g; mean brain weight NBW animals 106.86 g), but the relative brain an hippocampus weights, i.e. the weights expressed per kg body weight, of LBW animals were found to be higher in the LBW animals ($F_{1,8}$ = 6.15, P < 0.0047 and $F_{1,8}$ = 6.87, P < 0.0306 respectively).

Table 5. Performance in the cognitive pig holeboard. Differences between 4 treatment groups: Performance of LBW and NBW piglets born from allopurinol treated sows (n=6) and controls (n=4).

		Between subjects			**Within subjects**								
		Treatment (TM)			Blocks (Bs) (or Door*(D))			Birth weight (BW)			BW x TM		
Measure	Phase	F	df	P <	F	df	P <	F	df	P <	F	df	P <
Trial Duration	training	0.08	1,8	0.7877	18.59	9,72	**<0.0001**	0.04	1,8	0.8551	0.62	1,8	0.4536
	reversal	0.20	1,8	0.6698	90.13	5,40	**<0.0001**	0.07	1,8	0.8029	1.23	1,8	0.3129
Work. Memory ratio	training	0.04	1,8	0.8409	8.94	9,72	**<0.0001**	0.39	1,8	0.5503	1.10	1,8	0.3248
	reversal	0.32	1,8	0.5843	26.63	5,40	**<0.0001**	0.09	1,8	0.7725	0.04	1,8	0.8469
Ref. Memory ratio	training	0.42	1,8	0.5372	63.21	9,72	**<0.0001**	1.03	1,8	0.3392	0.35	1,8	0.5686
	reversal	0.46	1,8	0.5188	61.16	5,40	**<0.0001**	0.02	1,8	0.8859	0.27	1,8	0.6183
Inter Visit Interval	training	0.12	1,8	0.7043	7.06	9,72	**<0.0001**	0.04	1,8	0.8547	0.12	1,8	0.7385
	reversal	0.05	1,8	0.8223	32.08	5,40	**<0.0001**	0.07	1,8	0.7992	0.84	1,8	0.3861
Trials to Criterion	training	0.30	1,8	0.5981	*n/a*	*n/a*	*n/a*	2.90	1,8	0.1273	1.10	1,8	0.3245
Response Flexibility													
- TD	transition	0.85	1,8	0.3835	*n/a*	*n/a*	*n/a*	0.07	1,8	0.7949	0.34	1,8	0.5749
- WM	transition	0.33	1,8	0.5787	*n/a*	*n/a*	*n/a*	0.69	1,8	0.4306	1.22	1,8	0.3013
- RM	transition	1.18	1,8	0.3084	*n/a*	*n/a*	*n/a*	2.57	1,8	0.1477	0.09	1,8	0.7737
- IVI	transition	1.52	1,8	0.2530	*n/a*	*n/a*	*n/a*	0.15	1,8	0.7060	0.63	1,8	0.4490
Strategies													
- Block differences	training	0.14	1,7	0.7166	0.56	9,63	0.8211	0.17	1,7	0.6892	1.38	1,7	0.2789
- Door differences*	training	0.08	1,8	0.7813	1.29	3,24	0.3005	0.03	1,8	0.8649	0.38	1,8	0.5537

		Within subjects								
		BW x Bs (or BW x D^)			Bs x TM			BW x Bs x TM (or BW x D x TM^)		
Measure	Phase	F	df	P <	F	df	P <	F	df	P <
Trial Duration	training	1.62	9,72	0.1263	0.9	9,72	0.5300	1.16	9,72	0.3340
	reversal	0.18	5,40	0.9669	1.23	5,40	0.3129	0.27	5,40	0.9248
Work. Memory ratio	training	0.74	9,72	0.6666	1.03	9,72	0.4232	1.55	9,72	0.1469
	reversal	0.58	5,40	0.7165	0.09	5,40	0.9923	1.11	5,40	0.3682
Ref. Memory ratio	training	0.67	9,72	0.7364	0.33	9,72	0.9608	0.52	9,72	0.8552
	reversal	0.27	5,40	0.9259	0.54	5,40	0.7412	1.74	5,40	0.2226
Inter Visit Interval	training	1.15	9,72	0.3373	0.65	9,72	0.7501	0.52	9,72	0.8589
	reversal	0.21	5,40	0.9580	3.28	5,40	**0.0141**	0.29	5,40	0.9135
Strategies										
- Block differences	training	0.14	1,7	0.7166	0.56	9,63	0.8211	0.17	1,7	0.6892
- Door differences*	training	0.08	1,8	0.7813	1.29	3,24	0.3005	0.03	1,8	0.8649

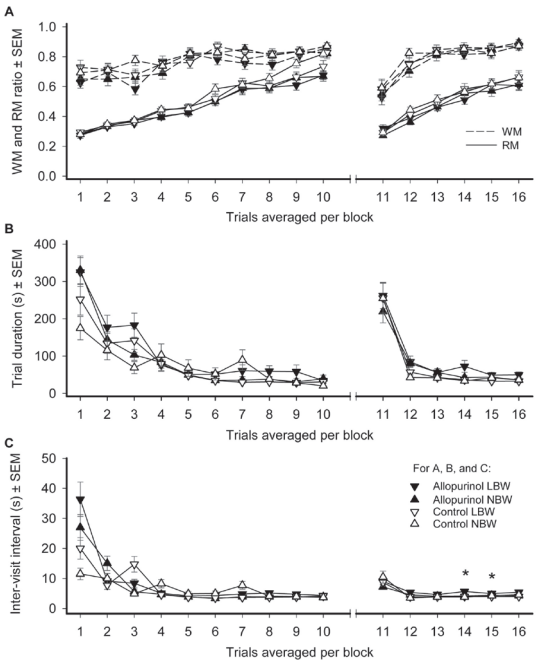


Fig. 3. Behaviour of four different treatment groups in a spatial holeboard task. Groups: low-birth-weight (LBW) and normal-birth-weight (NBW) piglets, prenatally treated with allopurinol (ALLO) and controls (CONT). Means and SEM for the ten trial blocks of the training phase (1-10) and six trial blocks of the reversal phase (11-16) shown for **(A)** WM and RM, **(B)** trial duration and **(C)** IVI. *Legend A, B and C:* ALLO LBW n = 10 piglets from 6 sows; ALLO NBW n = 10 piglets from 6 sows; CONT LBW n = 8 piglets from 4 sows; CONT NBW n = 9 piglets from 4 sows. RM = reference memory; WM = working memory

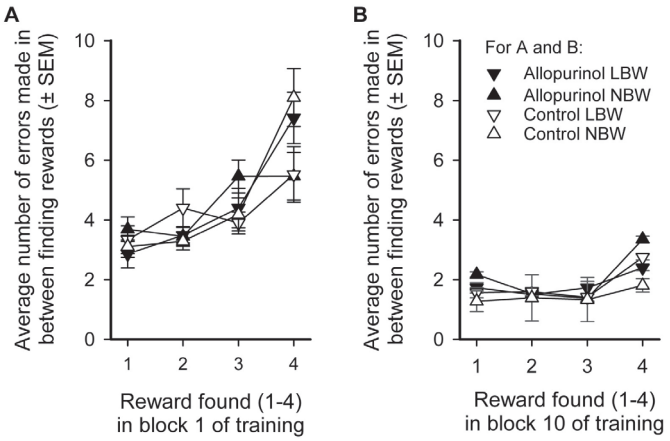


Fig. 4. Average number of errors* made per birth weight and treatment group between finding rewarded bowls (1: before locating the 1st reward, 2: between locating reward 1-2, 3: between locating reward 2-3, 4: between locating reward 3-4). Groups: low-birth-weight (LBW) and normal-birth-weight (NBW) piglets, prenatally treated with allopurinol (ALLO) and controls (CONT). Means and SEM for the 1st and 10th trial block** of the training phase are shown. Legend: ALLO LBW n = 10 piglets from 6 sows; ALLO NBW n = 10 piglets from 6 sows; CONT LBW n = 8 piglets from 4 sows; CONT NBW n = 9 piglets from 4 sows. **Error = visiting an unrewarded or previously rewarded bowl.**Trial block = 2 sessions of 2 consecutive trials.*

Table 6. A: Effect of birth weight, ALLO treatment and their interaction on brain weight and relative hippocampal and spleen weight (organ weight divided by body weight): The F-values, degrees of freedom and associated p-values are listed. **B:** Relative and absolute weights per birth weight by treatment group are listed as mean and standard error of the mean (SEM).

A	Between subjects			Within subjects					
	Treatment (TM)			Birth weight (BW)			BW x TM		
Measure	F	df	P <	F	df	P <	F	df	P <
End body weight	0.27	1,8	0.6164	5.20	1,8	**0.0047**	3.92	1,8	0.0832
Mean brain weight	0.19	1,8	0.6760	6.15	1,8	**0.0381**	0.08	1,8	0.7911
Mean hippocampal weight	0.65	1,8	0.4428	6.87	1,8	**0.0306**	0.33	1,8	0.5806
Mean spleen weight	4.23	1,8	0.0738	0.22	1,8	0.6546	0.14	1,8	0.7150

B	Absolute weights						Relative weights					
	Measure	Mean	SEM	Measure	Mean	SEM	Measure	Mean	SEM	Measure	Mean	SEM
End body weight	LBW ALLO	87.10	7.10	NBW ALLO	98.60	4.75	LBW ALLO	n/a	n/a	NBW ALLO	n/a	n/a
(kg)	LBW CONT	73.00	7.15	NBW CONT	103.78	3.47	LBW CONT	n/a	n/a	NBW CONT	n/a	n/a
Brain (g)	LBW ALLO	102.00	1.51	LBW ALLO	105.60	2.51	LBW ALLO	1.24	0.10	LBW ALLO	1.09	0.04
	LBW CONT	99.00	4.63	LBW CONT	108.11	2.85	LBW CONT	1.35	0.08	LBW CONT	1.05	0.05
Hippocampus (g)	LBW ALLO	3.18	0.06	LBW ALLO	3.30	0.09	LBW ALLO	0.039	0.0033	LBW ALLO	0.034	0.0015
	LBW CONT	2.86	0.27	LBW CONT	3.03	0.10	LBW CONT	0.042	0.0039	LBW CONT	0.030	0.0015
Spleen (g)	LBW ALLO	118.00	9.46	LBW ALLO	121.30	6.10	LBW ALLO	0.79	0.23	LBW ALLO	0.34	0.20
	LBW CONT	104.29	11.49	LBW CONT	131.78	6.98	LBW CONT	0.82	0.28	LBW CONT	0.68	0.22

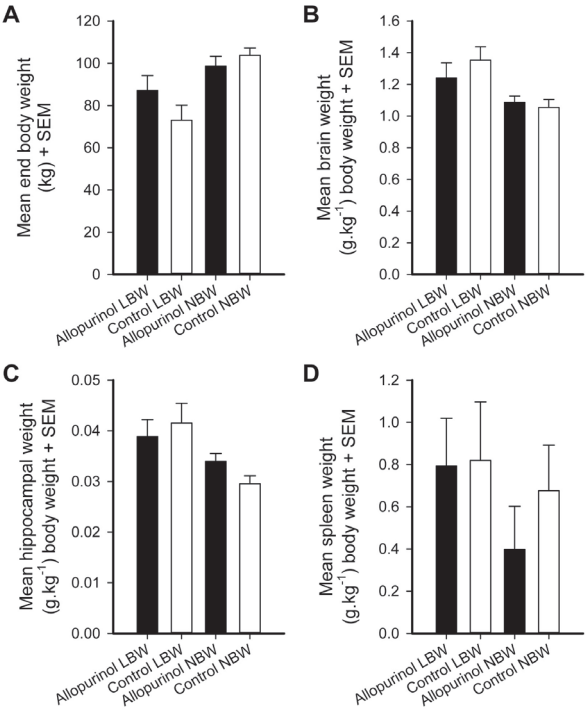


Fig. 5. Absolute body **(panel A)** and relative organ weights **(panels B-D)** of low- (LBW) and normal-birth-weight (NBW) pigs derived from allopurinol treated (ALLO) and control (CONT) sows at the age of 5 and 5.5 months. Ratios are calculated by dividing the organ weight through the end body weight per animal.

Discussion

The final aim of the present series of experiments was to assess the safety and efficacy of chronic prenatal oral treatment with ALLO of the sow on her unborn piglets. Mainly parameters related to cognition, emotional reactivity and growth were taken into account. Additionally also the progress of pregnancy and delivery including the placenta were registered. All sows were observed starting before the onset of parturition till the end of the weaning period. During delivery no complications occurred. The lengths of parturition fell within the normal range and all placentas were released in a natural way. The interpretation and implications of the findings will be discussed in this chapter one by one.

Pharmacokinetics of allopurinol

A brief pharmacokinetic study was performed with sows and their piglets to determine the oral dose reaching adequate neuroprotective plasma levels for ALLO and/or OXY in piglets, which were suggested to be >2 $\mu g.ml^{-1}$ for ALLO and >4 $\mu g.ml^{-1}$ for OXY (Torrance et al. 2009).

Neuroprotective plasma levels were reached for ALLO but not for OXY. Comparable results were found by (van Dijk et al. 2008), presumably because OXY molecules can hardly cross the placenta and/or neonate piglets are inable to convert ALLO into OXY in their foetal liver (van Dijk et al. 2008). Therefore it is unlikely that neuroprotective effects on the foetus are caused by OXY, and neuroprotective effects are expected at a plasma level $\geq$ 2 $\mu g.ml^{-1}$ ALLO. Our measurements allowed a simulation to estimate the daily dose that was necessary to reach steady therapeutic levels for neuroprotection.

Blood gas levels

Blood gases were measured immediately after birth to identify piglets that probably suffered from acute birth asphyxia. Allopurinol didn't seem to have influenced blood gas values of neonatal piglets prenatally treated from day 86 (+ 1-3 days) of gestation including the day of delivery. Neither did any of the sampled piglets seem to have suffered from acute asphyxia as no pH levels <7.0 or pCO2 levels >100 mm HG were measured (Belai et al. 1998, Randall. 1971, van den Berg et al. 1996). Though, these levels are generally based on venous or arterial blood samples compared to the mixed samples drawn in this experiment. Variation between pH and pCO_2 levels in mixed, venous or arterial blood of piglets is unknown. In total 35% of the piglets behaviourally tested were sampled for blood gas values. As blood samples couldn't be drawn from all piglets, this might implicate that an unwanted bias selected for piglets that were easy to sample and excluded others. Umbilical cords from specific sows seemed to break much easier compared cords of others, which often hindered blood sampling. Blood samples were drawn as soon as possible after delivery and cutting the umbilical cord, but gas exchange through breathing could not be prevented. This could have influenced the results (Randall. 1971). Some analysis of samples failed due to excessive air in the sample. Possibly the level of oxidative stress determined by blood gas parameters may in the future be substituted or completed by evaluating (anti-) oxidative parameters of placental tissue or maternal plasma (Biri et al. 2007).

Placental features and body measures

To assure that chronic ALLO treatment had no effects on general placental features, basic measures were taken from placentas of treated and control piglets. Most placental features were unaffected by prolonged ALLO treatment, except that placentas derived from treated piglets were found to be shorter. This contrasts with the finding that body length is longer in ALLO treated piglets. According to Wilson et al. (1998) placental size is inversely correlated with its efficiency, i.e. smaller placentas seem to be relatively more efficient. As the ALLO treated piglets are found to be taller but not heavier compared to controls, the differing placenta length does not seem to be a factor biologically relevant for the health and viability of ALLO treated piglets.

The correlations found between placental measures seem to be rational and correspond with previous findings (van Rens and van der Lende. 2002). Piglet birth weight is found to be correlated with two placental measures namely placental weight and circumference. These findings corroborate findings from van Rens and van der Lende (2002) and Rootwelt et al. (2012), but also findings from human studies of the placenta (McKeown and Record. 1953).

Open field and novel object test

To assess the effects of ALLO treatment on the anxiety level of the piglets, an open field and novel object test (combined) was performed just after weaning. Emotional reactivity of ALLO treated piglets in the open field test didn't seem to differ from that of the controls, neither in the LBW nor the NBW group. However, LBW piglets vocalised more than NBW piglets. Increased vocalisations in piglets are shown to be correlated with unpleasant or painful situations (Rushen. 2000) and social isolation (Donald et al. 2011), all known to be stressful and anxiety inducing events. Stress-reducing drugs as azaperone are found to decrease the number of vocalisations in piglets when subjected to a stressful environment (Donald et al. 2011). Piglets born from cortisol treated sows, a prenatal stress model, were found to vocalise more compared to controls in a novel environment test in a study by Kranendonk et al. (2006). Our finding that LBW piglets vocalise more in the open field test compared to NBW sibling corroborates findings of Weary et al. (1997), who found isolated LBW piglets to vocalize more compared to isolated NBW piglets.

Chronic ALLO treatment did not affect the increased anxiety levels in LBW piglets in any direction (mean frequency ± SEM; LBW ALLO: 318.80 ± 49.92; LBW CONT: 338.63 ± 30.29) but it was increased in the LBW compared to NBW animals. Epidemiological studies in (v)LBW children showed anxiety to be increased (Hayes and Sharif. 2009), which suggests that increased anxiety related to LBW is shared amongst humans and pigs.

Holeboard

The measures WM and RM clearly showed that all four groups were well able to learn both the initial configuration and a reversal of the holeboard task. These results are in line with earlier pig-holeboard studies (Arts et al. 2009, Gieling et al. 2012). However, one of our earlier studies (Gieling et al. 2012) showed LBW

animals to have more difficulty with the transition from one learned configuration to a new one (reversal), compared to NBW siblings. This difference in WM performance was not found in the current experiment. A methodological difference between the studies was the moment at which the reversal was commenced. In the experiment by Gieling et al. (2012) all pigs started the first reversal after 26 trials. The current experiment applied a RM performance criterion before switching to the reversal and a minimal number of 40 trials during the acquisition phase of the first configuration. Especially the response flexibility (see Fig. 4) of WM, RM and TD (end performance 1st configuration – start performance reversal) clearly shows that if all animals reached a minimal performance level before they start learning a new configuration, they have less difficulty switching. The general rules of the task (which are RM related (Koehl and Abrous. 2011)) might have been stored better after a longer training period, which makes switching easier. Another difference was that trials were presented once a day as a set of two massed trials. In the previous study two trials a day were given but they were spaced over the day. To be able to conclude if WM and RM performance under these different conditions truly differ, we analysed performance of the untreated LBW and NBW animals and compared it with the results of Gieling et al. (2012) after 26 training trials. WM and RM performance under the two given circumstances was found to be very similar. In the present study the sow was the unit of treatment and not the individual piglets. This causes a loss of statistical power and affects the sensitivity of tracing possible subtle behavioural differences between groups.

Human studies comparing cognitive performance of LBW children with healthy controls differ substantially for their setup. Not all of them found LBW (but term) born children to be affected by prenatal growth restriction later in life (Bos et al. 2001). Altogether we could speculate that not all LBW piglets are clearly cognitively affected by their growth restriction. A clear discrepancy between this pig study and human studies is that our piglets were kept under conventional farm circumstances which are not optimal for survival of the piglets most severely affected by their growth restriction. As survival rates of the most affected LBW children are going up in the western world (O'Shea et al. 1998), optimally this high level of neonatal care should be imitated in the translational pig studies to ensure inclusion of the most affected animals and avoid a bias through loss of the less viable animals.

Except for a treatment*block interaction effect on inter-visit-interval during the reversal phase, no learning and memory differences were found between the ALLO treated and control group. No conclusions can be drawn about the possible positive effect of prenatal ALLO treatment on the cognitive performance of LBW piglets. However, we also did not find any indication that ALLO had a detrimental effect on cognitive performance when piglets were tested from seven weeks of age.

Clearly more EpR were made during the 1st block of training compared to the last communal block of training (block ten). During the first block, the number of EpR increased with each reward still to be found in a trial, but no differences between treatment or birthweight groups were seen. During a later stage of training (block ten), the number of errors stayed more or less similar till the 3rd reward is

found. This measure reflects that 1) most animals reached a high, but not errorless performance level, and 2) that in particular from the 3rd reward onwards the task becomes more difficult for most of the pigs. The latter could be related to the attention span of the animals or their memory load capacities and is a fact to keep in mind when defining the difficulty level of a learning task. Although attention span is found to be impaired in (v)LBW children (Shum et al. 2008), this was not confirmed in our LBW piglets by the EpR analysis.

As for the CC scores, no treatment effects of ALLO were found. Both BW groups were found to apply a partial strategy per specific entry door, but no clear development of a search strategy was seen over blocks, when 'door' was not included in the analysis. All CC scores per door clearly differ from the random performance score 1.72, calculated over all training trials of both BW groups (Bouger and van der Staay. 2005). As average CC scores are found to be 2.263 (±SEM 0.139) for the LBW and 2.257 (±SEM 0.131) for the NBW animals, it is clear that the animals adopt a search pattern per entry door, although optimal performance (a score of 4) was never reached. As these measures were calculated over all training trials with a specific entry door, scores are lowered by the initial trials during which the animal still had limited information about the rules of the task (RM related information) and the location and number of rewards. Therefore ultimate performance should be calculated with later trials only. The pigs did not develop one specific strategy if the analysis was run across all entry doors. However, if entry door was considered in the analysis, it became obvious that the search pattern differed per entry door. This shows most pigs are able to develop a (partial) strategy per entry door. This accounts for both BW groups and strengthens the idea that having more than one entry door increases the difficulty of the task (Arts et al. 2009).

Brain, spleen and body weight

The LBW piglets did not show compensatory weight gain and their final body weight was still lower compared to the average weight of NBW animals at slaughter. Lasting effects on body weight are in agreement with previous studies (Powell and Aberle. 1980, Rehfeldt and Kuhn. 2006) and are also observed in human LBW children (Xiong et al. 2005). Additionally a marginal treatment*birthweight interaction effect was found. The data suggests that the birth weight of ALLO treated LBW piglets is higher compared to that of untreated LBW animals and that this effect of ALLO did not occur in the NBW groups. This effect is not reflected by the relative brain and hippocampus weights of the animals. The findings are in agreement with the data from LBW children that remain atypically small during early years and have greater chances of less than optimal cognitive development (Casey. 2008). Therefore postnatal growth is an important developmental factor.

Spleen weights (corrected for body weight, as spleen weight increases with body weight (DeLand. 1970)) of ALLO treated animals tended to be lower than those of controls. In particular, the relative spleen weights of ALLO treated but NBW animals tended to be lower than those of the other three groups. A characteristic of chronic stress (as we suppose has occurred during the prenatal period of LBW piglets) is a change of size in stress-related tissues (Blanchard et al. 1995).

Long-lasting stress is known to decrease the weight of organs such as the spleen (Hara et al. 1981, van der Staay et al. 2010, Tuli et al. 1995), but this involution of the organ is also found to eventually return to normal after termination of stress (Marsh and Litwack Jr. 1960). On the contrary, Blanchard et al. (1995) found spleen weights corrected for body weight to increase in chronically stressed animals, but this may reflect an inflammatory response to wounding as male dominance hierarchies were studied in their experiment. Furthermore, the actual organ weight is largely determined by the actual blood flow and erythrocyte storage into this organ (Laidley and Leatherland. 1988). It would be premature to indicate whether and how stress levels were influenced by ALLO treatment in one of the BW groups based on this marginal finding and discrepancies in literature.

Neither relative brain nor hippocampus weights were influenced by ALLO treatment in any direction. Both measures are found to be higher in LBW compared to NBW animals, while absolute weights are lower. In preterm (v) LBW children, lower brain and hippocampal weights were found (Isaacs et al. 2000). In human children, head circumference (a measure correlating to brain weight) at eight months of age appears to be the best growth parameter for predicting IQ at the age of three years. Adequate compensatory brain growth during the first year of life could prevent much of the negative effects on IQ at three years of age. (Hack and Breslau. 1986) The brains of the pigs in this study were weighed at 5-5.5 months of age. Probably because the LBW piglets selected suffered from relatively mild IUGR, partial compensatory postnatal brain growth could have taken place.

Conclusions

The aim of this study was to assess both safety and efficacy of prolonged prenatal oral ALLO treatment in piglets via the sow. Preliminary analysis of the plasma concentrations in sows and their piglets suggested that a daily dose of 15 $mg.kg^{-1}$ results in effective plasma concentration of ALLO in piglets. In contrast to studies with other animal species as well as humans, only relevant ALLO but not OXY levels were measured in the unborn/neonatal piglets and no accumulation of the drug was measured in the sows.

ALLO treatments, even over a slightly longer period, had no adverse effects on farrowing, confirming previous findings in pigs by Boda et al. (1999). These authors applied a dose of 30 $mg.kg^{-1}$ during 4-8 days preceding delivery. In the present study, none of the piglets sampled showed blood gas values indicating that they had suffered from acute birth hypoxia.

The placental features ‘weight’ and ‘circumference’ were found to correlate with piglet birth weight. ALLO treated piglets seemed to have shorter placentas. As the treated pigs were also found to have taller bodies, placenta length does not seem to be a naturally relevant factor influencing the growth of treated piglets. No interaction effects between treatment and birth weight were found.

An open field test for emotional reactivity at five weeks of age did not reveal any differences between treated and untreated animals. Though, LBW animals were found to vocalize more compared to NBW siblings. Their anxiety levels are

probably increased, as is found for LBW human children. We therefore suggest it to be a shared phenomenon amongst both humans and pigs.

Evaluating the cognitive capacities of ALLO treated piglets in the cognitive holeboard task we could not identify an effect of the ALLO treatment. Neither were there any differences found between LBW and NBW piglets. These findings contrast with the results of a previous study in which we observed differences in response flexibility between LBW and NBW piglets after switching to a new configuration (Gieling et al. 2012). This discrepancy might be attributable to the fact that the experimental unit differed between both experiments (eventually affecting statistical power and sensitivity). Also in the present study we trained animals until a specific (higher) level of performance was reached. However, results clearly indicated that a prolonged prenatal treatment with ALLO can be regarded as safe as no undesirable side effects were observed.

LBW piglets did not reach the same final body weights as NBW animals, but body weight at 5-5.5 months of age showed a certain level of postnatal compensatory growth, as did brain and hippocampus. LBW animals treated with ALLO showed the largest postnatal compensatory body weight gain, which is a positive indication for the chronic prenatal use of ALLO in these animals. Further research should take into account that relative spleen weights tended to be lower in treated NBW animals, although relative brain and hippocampus weights were not influenced by treatment.
We conclude that prolonged prenatal ALLO treatment during the third trimester in sows and their LBW and NBW piglets tends to be safe during pregnancy and delivery, and did not affect the postnatal period. The efficacy of treatment on the cognitive performance of the piglets remains unclear, despite the fact that the plasma-concentrations time curves measured in sows and also the piglets confirmed the diaplacental transfer of ALLO reaching steady state concentrations (Graham et al. 1996) which are believed to be therapeutically active. Relative brain and hippocampus weights seem to be unaffected by treatment but the final growth of treated LBW pigs appears to be improved compared to the other three groups.

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Effects of prenatal allopurinol treatment on brain plasticity markers in low- and normal-birth-weight piglets

Submitted manuscript

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Abstract

Children born with a low-birth-weight (LBW) are at risk for several diseases, even if carried to term. Possible consequences of being born with a LBW are for example learning problems, a higher risk for depression and a reduced brain volume. Placental insufficiency leading to hypoxemia and reduced nutritional supply is the main cause for LBW. One of the consequences of an oxygen deprived brain is compromised neuronal activity. Therefore, in this study we investigated in piglets the effect of low (LBW) and normal (NBW) birth weight on protein levels of several plasticity markers in the dorsal hippocampus. Furthermore, we tested if Allopurinol (ALLO), a xanthine oxidase inhibitor, which is clinically given to asphyxiated neonates, has a protective effect on these plasticity markers. Sows were treated daily with ALLO in the last trimester of pregnancy. Our results show no significant birth weight effect on the tested plasticity markers. However, we found that ALLO increases protein levels of brain-derived neurotrophic factor (BDNF) and the postsynaptic marker PSD95 in low and normal-birth-weight piglets. Analysis of the transcription factor pCREB/CREB ratio revealed that ALLO treatment seems to increase the ratio in LBW pigs, while having an opposite effect on the ratio in NBW pigs. We found neither treatment nor birth weight effect for synaptophysin, TrkB, ERK2 and the pGSK/GSK ratio. Taken together, ALLO might be a promising treatment of the adverse effects of LBW on the brain.

Introduction

In the United States (US), 8% of the children are born with a low-birth-weight (LBW) after being carried to term(small for gestational age (SGA)) (2002-2003) (Ergaz et al. 2005). LBW children are at risk for several diseases, neonatal mortality and morbidity (Gagnon. 2003). Risk factors are more severe if LBW is combined with being born preterm (Chaudhari et al. 2004). Chaudhari et al. (2004) showed that children with LBW had a lower IQ, decreased visuo-motor perception, reduced motor competence and poorer academic achievement compared to children with a normal-birth-weight (NBW) at an age of 12 years. LBW after being carried to term gives better chances for survival. However, term born LBW children are still at risk for developing several health problems, as well as cognitive deficiencies such as (general) learning problems (Chaudhari et al. 2004). They also have an increased risk for depression (Raikkonen et al. 2008), and a may suffer from a reduced brain volume (Martinussen et al. 2009, Toft et al. 1995).

Possible causes for LBW are various and can be well-defined (i.e. chromosomal disorders, intra uterine viral infections) (Albertson-Wikland et al. 1993) or ambiguous (i.e. smoking, air pollution or placental insufficiency) (Ergaz et al. 2005). A known consequence of an oxygen deprived brain is compromised neuronal plasticity resulting in neuronal cell damage or even death (Li et al. 2011). In general it can be said that a foetus suffering from hypoxemia or reduced nutritional supply will slow down its growth rate (Gagnon. 2003). Pharmacological treatment with neuroprotective substances, preventing the formation of, or scavenging the free-radicals produced, could possibly improve neurological outcome in these cases.

Allopurinol (ALLO), a free-radical scavenger, is a xanthine oxidase inhibitor which together with its active metabolite oxypurinol (OXY) acts as a scavenger for toxic hydroxyl free radicals and chelates non-protein-bound (pro-radical) iron (Moorhouse et al. 1987). ALLO has been found to reduce free-radical formation in for example pig and human foetuses (Boda et al. 1999). In the clinics, ALLO is currently only acutely administered in asphyxiated neonates during or after birth, possibly providing neuroprotective effects. Nowadays pregnant woman in developed countries are monitored throughout their pregnancy, and growth restriction due to placental insufficiency can reliably be diagnosed during the second or third trimester of pregnancy. However, preventive therapies are not yet available (Sankaran and Kyle. 2009).

Comparable to undersized human neonates, LBW piglets show characteristics of immaturity/dysmaturity. High fecundity in sows and consequent increases in litter size in commercial pig farming have resulted in more piglets being born with LBW in each litter. Within sow comparison between term carried LBW piglets and their littermates of normal-birth-weight (NBW) is considered to be an appropriate model to study effects of birth weight on later development (Gieling et al. 2011). Recently we showed that LBW piglets display transiently retarded learning in reversal learning in the holeboard discrimination task (Gieling et al. 2012).

Responsiveness to induced hypoxic insults was recently monitored in neonatal mice, a model corresponding to the hypoxic insults observed in the very low-birth-

weight premature infant population. Brain plasticity markers, in particular the brain-derived neurotrophic factor (BDNF) signalling pathways, were significantly reduced whereas hypoxic markers were increased. (Li et al. 2011)

In the present study, we investigated whether prolonged prenatal ALLO treatment during the last trimester of pregnancy improves neuronal plasticity in LBW pigs compared to NBW pigs, thereby limiting later cognitive deficits in life. ALLO readily crosses the human and pig placenta and does not interfere with the delivery process if administered acutely during parturition (Boda et al. 1999). Although generally less severe than asphyxia, the birth weight comparison paradigm is an interesting model to study prenatal ALLO treatment of term born LBW pigs. Dorsal hippocampal brain tissue of 5-5.5 month old pigs was investigated for expression of relevant markers in the BDNF mediated neuronal plasticity pathway.

Material and Methods

Ethical approval

The experiments were reviewed and approved by the local ethics committee at Utrecht University, and were conducted in accordance with the recommendations of the EU directive 86/609/EEC. All effort was taken to minimize the number of animals used and their suffering.

Animals and housing

Sows: Twelve pregnant sows (divided over two batches of six sows, with two months between batches) were kept in a group housing system for sows with automatic feeders, an outside area where silage was provided, straw bedding and *ad libitum* access to water. The ambient inside temperature ranged between 15 and 25°C and light was provided between 07:00h and 22:00h. Except for the pellets mixed with the treatment (1 kg), the daily food ration (standard pregnant sow pellets, de Heus, Ede, the Netherlands) was distributed via an automatic feeder. One week prior to the expected farrowing date all sows were moved to a conventional farrowing stall [ambient temperature 20-23°C, floor heating 30°C (piglet area only)] and were housed there till the piglets were weaned. Food was provided automatically two times a day and access to water was *ad libitum*.

Treatment of piglets via sows: From twelve pregnant sows (see Table 1), six were treated with ALLO (15 $mg.kg^{-1}$, based on a previous pharmacokinetic study, see chapter six) for 30 days (±2 days depending on the actual farrowing date, starting at day 86 of pregnancy) and six were used as control subjects. ALLO tablets (300 mg, Ratiopharm, the Netherlands) were powdered and mixed with 1 kg of pellets, some honey and water. Animals were observed till all the food was consumed. Sows were weighed weekly to adapt the dose once a week to their weight gain or loss. The last dose was administered on the day of farrowing.

Piglets: After taking all birth measures upon natural delivery, piglets returned to the sow directly to drink colostrum. Starting at 2-3 days of age extra artificial milk for piglets (Milkiwean, Trouw Nutrition, the Netherlands) was provided in a drinking bowl in the pen. At 3 days of age all piglets were preventively given an iron injection. When birth diarrhoea occurred, all piglets from the affected litter

were treated orally with colistine (Enterogel, Virbac Animal Health, Barneveld, the Netherlands) for 3-5 days. Crippled piglets before or after weaning where treated with ampicillin (Ampicillan 20%, Alfasan Nederland B.V., Woerden, the Netherlands) for 3-5 days and if necessary an analgesic with meloxicam was administered once (Novem 20 mg.ml-1, Boehringer Ingelheim Vetmedica GmbH, Ingelheim, Germany). No tail docking or castration was applied in the selected LBW and NBW piglets.

After the piglets had reached 3.5-4 weeks of age the sow was removed and the piglets were weaned. After 1-1.5 more week(s) in the farrowing pen the selected LBW and NBW piglets were mixed and moved to two adjacent pens in the experimental unit.

Grouping

The average litter weight and the accompanying standard deviation (SD) were determined per litter. As described in Gieling et al. (2012), piglets weighing at least the mean litter weight minus 1x the SD were classified as LBW. After excluding all LBW piglets from the litter a new mean was derived. Animals with a weight closest to this new mean and with the same sex as the LBW animal(s) from the litter were selected as NBW animals. One to three LBW and one to three NBW animals were selected per litter, depending on availability. In this experiment each sow was considered as an experimental unit with or without ALLO treatment (between subjects factor). NBW and LBW piglets were considered as within subjects factor. Therefore, the average of all NBW and LBW piglets per sow was applied for analysis.

Table 1. Overview of the sows and piglets used for the experiment. All animals are a (Terra x Finnish landrace) x Duroc mix.

Animals	Group	Parity	Litter size	Av. Lit.	♂/♀	LBW and NBW piglets selected for testing					
Batch 1						LBW (gender/ birth weight)			NBW (gender/ birth weight)		
Sow 1	ALLO	7	14 (+2†)	1303.21	10/ 4	♂/ 755g	♂/ 1040g		♂/ 1300g	♂/ 1375g	
Sow 2	ALLO	4	13 (+4†)	1276.54	7/ 6	♀/ 890g	♂/ 980g		♀/ 1400g	♀/ 1480g	
Sow 3	ALLO	2	12 (+2†)	1275.83	5/ 7	♂/ 875g			♂/ 1410g		
Sow 4	Control	6	18 (+1†)	1362.67	11/ 7	♂/ 715g	♂/ 720g	♀/ 1080g	♂/ 1660g	♀/ 1710g	♂/ 1750g
Sow 5	Control	4	17	1338.82	13/ 4	♂/ 930	♂/ 1070g	♂/ 1085g	♂/ 1370	♂/ 1440g	♂/ 1340g
Sow 6*	Control	3	10	1855.50	7/ 3	-	-	-	-	-	-
Batch 2						LBW (gender/ birth weight)			NBW (gender/ birth weight)		
Sow 7	ALLO	9	9 (+4†)	1607.79	5/ 4	♂ 1155			♂/ 1740		
Sow 8	ALLO	2	18	1439.44	11/ 7	♀ 860	♂/ 1115		♀/ 1525	♂/ 1700	
Sow 9	ALLO	2	12	1546.67	6/ 6	♀ 1040	♀/ 825		♀/ 1665	♀/ 1590	
Sow 10	Control	8	8	1304.38	7/ 1	♂ 870			♂/ 1415	♂/ 1460	
Sow 11*	Control	2	5	2028.00	2/ 3	-	-	-	-	-	-
Sow 12	Control	10	19	0858.89	12/ 7	♂ 470			♂/ 950		

*No piglets from these litters were selected, as no piglets could be classified as LBW animals according to the criteria outlined above.

Collection of organ tissue

At the age of 5 (batch 1) or 5.5 months (batch 2) the pigs were transported to a local slaughterhouse, where they arrived about 24 hours before slaughtering. They stayed in a pen as a group (i.e. no mixing with other animals), and entered the lairage, only a few meters from the pen, approximately 30 minutes before slaughtering started. The order of slaughter was randomized. The first pig was slaughtered at approximately 12:00 and the last pig was killed about 4 ½ hours later.

A pig was stunned with an electrical stunner, bled and immediately decapitated. The brain was rapidly excised and the hippocampi were dissected. They were transversely cut into three equal parts: the ventral and dorsal samples were rapidly frozen per hemisphere in liquid nitrogen. The intermediate third was not used. All brain samples were stored on dry ice until they were transferred to a freezer where they were stored at −80 °C until further processing.

Tissue preparation

100-130 mg dorsal hippocampus tissue was homogenized using a Mini-Bead Beater (Biospec products, Bartlesville, USA) three times for 30 s in 1 ml ice-cold homogenizing buffer (100 mMTris, 200 mMNaCl, 1 mM EDTA, 2 mM DTT, 0.05% Triton vol/vol and a phosphatase inhibitor tablets/10 ml (Roche #04906837001, Vilvoorde, The Netherlands and a protease inhibitor tablet/20 ml (Roche #11836153001, Vilvoorde, The Netherlands). Samples were cleared by centrifuging for 20 min at 4°C, 16000g and the supernatant was stored at -80°C. Protein concentrations were determined using Bio-Rad Lowry Protein Assay (Bio-Rad Laboratories inc., Hercules USA).

Western Blotting

Brain homogenates inhomogenizing buffer were boiled for 7 min and then separated on a 7,5% (TrkB), 10% (pCREB, CREB, PSD95,pGSK, GSK, ERK2, Synaptophysin)or 14% (BDNF) SDS–PAGE gel (10 µg protein/sample: Synaptophysin, 25 µg/ sample: PSD95, ERK2; 30 µg/sample:pCREB, CREB, pGSK, GSK, BDNF; 50 µg/sample:TrkB). Following electrophoresis, proteinswere transferred to a nitrocellulose membrane (Bio-Rad Laboratories,Hercules, USA) which was subsequently blocked with blockingbuffer (50% Odyssey blocking buffer in PBS, Li-Cor, Lincoln, USA)for one hour at room temperature. Next, the membranes were incubated overnight at 4°C with the primary antibodies in blockingbuffer: 1:500 rabbit anti-BDNF (H-117) (Santa Cruz Biotechnology#12811, Santa Cruz, USA), 1:250rabbit anti-TrkB (Cell Signaling Technology #9104, Beverly, USA), 1:3000 mouse anti-CREB (Cell Signaling Technology #9104, Beverly, USA), 1:100 rabbit anti-pCREB (Cell Signaling Technology #9198S, Beverly, USA), 1:1000 rabbit anti-GSK-3β (Cell Signaling Technology #9315S, Beverly, USA), 1:1000 rabbit anti-pGSK-3β (Cell Signaling Technology #9336S, Beverly, USA), 1:2000 mouse anti-PSD95 (QED Bioscience inc #0711, San Diego, USA), mouse anti-Synaptophysin (Millipore MSxsynaptophysin MS #MAB5258, Billerica, USA), 1:1000 rabbit anti-ERK2 (Cell Signaling Technology #9108, Beverly, USA),with 1:1000 β-Actin (Santa Cruz Biotechnology #12811, Santa Cruz, USA) or 1:2.000.000mouse anti-GAPDH (#10R-G109A, Fitzgerald, Huissen, The Nether-lands) for normalization. After washing with phosphate-buffered saline-

0.1%Tween (PBS-T), membranes were incubated for 1 hour at room temperature with secondary antibodies in blocking buffer: 1:5000 goat anti-rabbit IRDye 800 (#926-32211, Li-Cor) and 1:10000 donkey anti-mouse IRDye 680 (#926-32222, Li-Cor). Membranes were washed in PBS-T and fluorescent bands were visualized using an Odyssey Infrared Imaging System (Li-Cor). Intensities of specific bands were quantified using ImageJ (http://rsbweb.nih.gov/ij/), corrected for background signal and β-Actin or GAPDH signal.

Analysis

The data was analyzed with SAS 9.2. NBW and LBW piglet data was averaged per sow (allopurinol or control). Significance level was fixed at ≤ 0.05. First it was tested if the data was distributed normally using a Shapiro-Wilk test. If this was the case a Repeated Measures Analysis of Variance (SAS GLM procedure) was performed to look at the effect of ALLO treatment, effects of birth weight, and the interaction between treatment and birth weight. If a measure was not normally distributed, a check for outliers was performed (http://www.graphpad.com/quickcalcs/Grubbs1.cfm). After exclusion of the outlier each measure was checked for normality again. If the data, after exclusion of outliers, still did not meet the prerequisite of normality, the data was log10 transformed.

Results

Western Blotting

BNDF: Analysis of the mature BDNF concentrations of the dorsal hippocampus demonstrated a significant ALLO treatment effect on LBW and NBW pigs ($F_{1,7}$=16.93, p=0.0045; see Fig. 1B). BDNF concentration was increased in the treated animals compared to the untreated animals of the same birth weight group.

PSD95: We observed a significant effect of ALLO treatment for PSD95 ($F_{1,7}$=7.79, p=0.0269; see Fig. 1F). PSD95 protein level was higher in ALLO treated animals compared to untreated animals. No birth weight effect was detected.

pCREB/CREB: A marginal birth weight effect by ALLO treatment interaction for the ratio of pCREB and CREB was found ($F_{1,8}$=4.1, p=0.0801; see Fig. 1C). LBW animals treated with ALLO had a marginally higher pCREB/CREB ratio untreated LBW pigs whereas ALLO treated NBW pigs had a decreased pCREB/CREB ratio compared to untreated NBW pigs.

pGSK/GSK, Synaptophysin, ERK2, TrkB: No effects of treatment or birth weight were found for the mBDNF high affinity receptor TrkB, Erk2, Synaptophysin and the pGSK-3β/GSK-3βratio (see Fig. 1A, Fig. 1D-E and Fig. 1G).

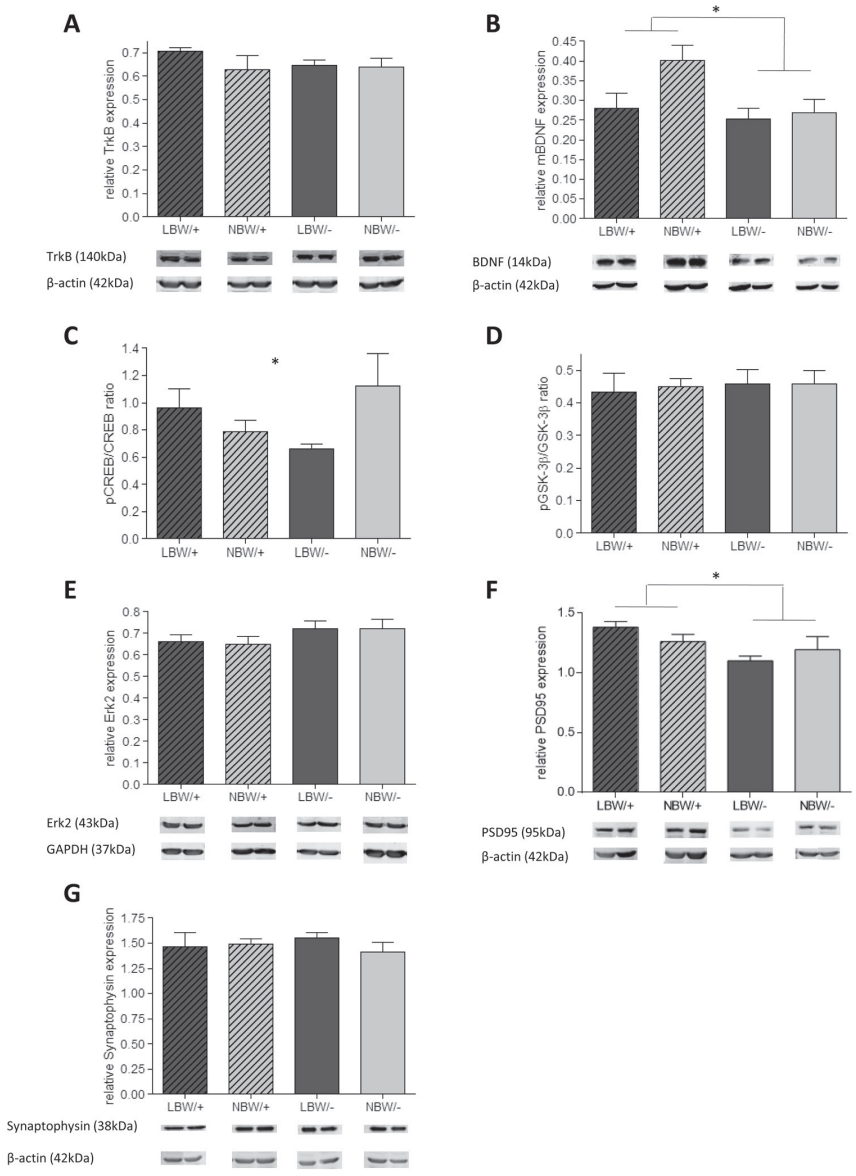

Fig. 1: Protein levels of ALLO treated LBW and NBW piglets in the dorsal hippocampus. ALLO treated LBW and NBW animals have significantly more hippocampal BDNF (A) and PSD95 (B) as compared to untreated animals. pCREB/CREB ratio shows an interaction tendency between treatment and birth weight (C). ALLO treated LBW animals have an increased pCREB/CREB ratio as compared to untreated LBW animals. ALLO treated NBW animals have a decreased pCREB/CREB ratio compared to untreated LBW animals. No differences in pGSK-3β /GSK-3β ratio (D), Erk2 (E), Synaptophysin (F) and TrkB (G), are observed. Bars indicate means ± SEM per group. LBW/+: low-birth-weight, ALLO treated; LBW/-: low-birth-weight, untreated; NBW/+: normal-birth-weight, ALLO treated; NBW/-: normal-birth-weight, untreated

Discussion

In this study, we observed significantly increased BDNF protein levels as well as increased PSD95 protein levels in ALLO treated LBW and NBW pigs compared to the untreated animals. Analysis of the pCREB/CREB ratio revealed a marginal birth weight by ALLO treatment interaction. ALLO treatment seemed to increase this ratio in LBW pigs, but to decrease the ratio in NBW animals. We found no treatment or birth weight effect for ERK2, Synaptophysin, TrkB and the pGSK/GSK ratio.

Previously, we showed LBW piglets to have more difficulty with the transition from one learned configuration to a new one (reversal) in the cognitive holeboard task, compared to NBW siblings (Gieling et al. 2012). This supports the hypothesis that LBW is related to mild cognitive impairments. Therefore, LWB piglets might be suitable animals to model LBW in human neonates and to study putative therapeutics which alleviate the negative consequences of SGA. Since the dorsal hippocampus plays an important role in memory formation, we focused our research on this part of the brain to determine the effects of LBW and ALLO treatment on plasticity markers.

Because of the size and brain development and the high similarity to humans, pig models yield results of high translational value (Gieling et al. 2011). However, we found no birth weight effect on any of the tested protein levels. The pigs have been handled and challenged regularly in the time between birth and sacrificing at an age of 5-5.5 months. It is known from a large number of rodent studies that environmental enrichment and training are able to reverse effects of an impoverished environment (Mohammed et al. 2002). Additionally, due to the drug treatment via the sow, and consequently the sow as experimental unit, it is difficult to perform a high power study. Despite these methodological difficulties, we found differences in PSD95 and BDNF protein levels upon prophylactic anti-oxidant treatment with ALLO. These results are in line with the study where mice which are rather resilient to hypoxic insult had an increased BDNF expression compared to mice which are rather prone to damage upon a hypoxic insult (Li et al. 2011). This could point to a possible relationship between anti-oxidants and a protective role modulated BDNF in this process.

Acute ALLO treatment was previously shown to have neuroprotective effects in animals and human neonates when administered to asphyxiated neonates during or immediately after birth (van Bel et al. 1998). Foetuses suffering from placental insufficiency are exposed to a longer, but probably milder lack of oxygen. We hypothesized that a chronic prophylactic treatment with ALLO may be more adequate. As mentioned above, we showed that ALLO treatment during the last trimester of pregnancy increased BDNF and PSD95 protein levels. The neurotrophin BDNF plays a critical role in long-term synaptic plasticity. It can develop and mature synapses and acutely modify synaptic efficacy (Schinder and Poo. 2000). PSD-95 is involved in orchestrating excitatory synapse maturation and specificity (El-Husseini et al. 2000). These results further support the hypothesis that ALLO has neuroprotective efficacy. This is of special interest for LBW piglets, as ALLO might be able to reduce the cognitive problems of

LBW newborns later in life (Chaudhari et al. 2004). Moreover, we observed a marginal ALLO treatment by birth weight interaction for the pCREB/CREB ratio. ALLO treatment increased the pCREB/CREB ratio in LBW pigs to a similar level as the ratio in untreated NBW pigs. However, it decreased the ratio in NBW pigs. If activated by phosphorylation, the transcription factor CREB induces the expression of a wide range of proteins including BDNF. pCREB is required for a variety of complex forms of memory, including spatial memory (for review see Silva et al. (1998)). It might be a topic for follow up studies to investigate if the increase in the pCREB/CREB ratio due to ALLO treatment is beneficial for the LBW piglets.

In conclusion, we did not find any effects of birth weight for the measured plasticity markers. Yet, treatment with ALLO increased mBDNF and PSD96 protein levels. Therefore, ALLO might be a promising treatment of the aversive effects of LBW.

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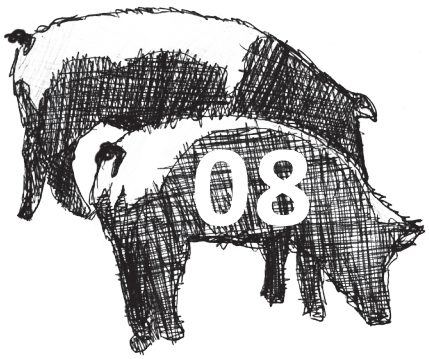

General discussion

Introduction

Being born with a restriction in prenatal growth may have long-term negative effects on an individual's functioning. This holds for human newborns as well as for piglets. In humans, cognition is one of the areas shown to be affected by intra uterine growth restriction (IUGR) (i.e. Cox and Marton. 2009, Hack. 2006, Martinussen et al. 2009). These cognitive effects may be modelled by appropriate animal models, such as the pig with prenatal growth restriction. The final aim of this thesis was to study the cognitive performance of piglets suffering from IUGR and to investigate the effects of a putative prophylactic treatment. Piglets are expected to provide a translational model for human medicine. In addition, these animals may yield new insights in the cognitive effects of IUGR that benefit the welfare of the farmed pig itself.

The final aim was to study the safety and efficacy of chronic treatment with the putative neuroprotective compound allopurinol (ALLO) in IUGR piglets. It was expected that ALLO prevents the possible adverse consequences of IUGR on the brain and on the later cognitive performance in IUGR animals. To be able to reach this aim it was necessary to perform a series of preparatory experiments: (1) to develop and validate a suitable cognitive test, (2) to learn about the pharmacokinetics of ALLO for treating IUGR foetuses during the last trimester of pregnancy, and (3) to investigate the effect of IUGR on the cognitive performance of piglets. These experiments and their main findings will be summarised and discussed in this chapter.

Test development

To be able to study cognition in the pig we developed two test setups suitable for pigs of various ages, starting early after weaning. To recapitulate from chapter 2, behavioural tasks for pigs should fulfil several criteria. They should be suitable for healthy and unimpaired animals, allow a detailed behavioural analysis, be as stress-free as possible, tap ecologically relevant behaviours, be standardised and automated, allow the investigation of developmental effects and repeated testing and should be complex and sensitive enough to detect subtle differences (Gieling et al. 2011).

In chapter 3 a simultaneous discrimination task was developed and applied to investigate whether pigs are able to discriminate between simple black and white symbols and between 2D images of conspecifics (an ability that had been documented for other (farm) animal species (Coulon et al. 2009, Kendrick et al. 1996). This test fulfilled several of the above mentioned criteria but did not allow a detailed behavioural analysis. Behaviour measured in this test consisted of a choice that could only be correct and rewarded or incorrect and unrewarded. As it is unknown whether pigs rely mainly on visual discrimination in daily life, it is questionable whether the test measures ecologically relevant behaviours. Results showed juvenile pigs to be able to discriminate between simple geometric symbols based on their vision, but not between faces of conspecifics (at least not during the large number of training trials given). We expected that ecologically relevant learning would be fast. The results suggest that the face discrimination task was too difficult, and/or that the pigs were insufficiently motivated to acquire

the discrimination or that it exceeded their mental abilities. Behaviour – all that an animal does in relation to the external world – is determined by several processes among which motivation, but also reflexes, modal action patterns, emotion and cognition (Toates. 2004). The cognitive decision a pig makes in the test is therefore influenced by different processes. Motivation could have been influenced by the difficulty level of, but also by the details and routine of the task. In this two-choice task, the chance of receiving a reward based on random choice was fairly high (50%). This may have been sufficient to maintain random choice behaviour. It took many trials to learn to attend to the visual stimuli presented and to start visual discrimination. Each session contained multiple trials which meant that after every trial the animal had to return to the start position. Moving away from the reward-area seemed to be frustrating for most pigs, especially after making an incorrect choice. This could have been stress-inducing and/or distracting and could have decreased motivation to perform. Some pigs refused participation halfway through a session. This session then had to be continued later on.

Reversal learning in the simultaneous discrimination task proved to be too demanding for many pigs. A performance criterion was applied before animals were allowed to switch from the original problem to the reversal. The reversal of a well-learned discrimination causes temporary confusion. If the general rules of the task are not understood well at the moment of switching to the reversal task, this could have caused more confusion or even frustration and thereby a decrease of motivation to perform. Understanding the general rules of a task is generally said to be part of the reference memory (RM; van der Staay et al. (2012)). RM is a well-defined measure in the cognitive holeboard task, whereas the simultaneous discrimination task does not yield a specific index of RM. Instead, discrimination tasks often set a learning criterion of a certain number of correct choices within a preset number of trials or sessions (e.g. Coulon et al. 2009, Ferreira et al. 2004). This type of criterion might relate more to the working memory component (WM; within-trial or session memory (van der Staay et al. 2012) instead of RM of an animal, and may therefore not be a good criterion to use when defining the moment of switching to a reversal. It is problematic to differentiate between the motivation and the cognitive ability to learn the simultaneous discrimination task. This should be studied before drawing final conclusions about the usefulness of this task for specific applications such as, for example, discriminating between pictures of conspecifics. Introducing a penalty contingent on a wrong decision could increase the urge to choose thoughtfully instead of choosing randomly, which yields already at 50% chance to gain the reward. However, introducing a penalty in pigs usually also introduces a certain level of stress in the test which could interfere with cognitive performance (Mendl. 1999, Schwabe and Wolf. 2010). Additionally it could decrease motivation 1) out of fear of the penalty (depending on the type and severity of penalty) or 2) because the combination of a high difficulty level plus a punishment after an incorrect choice makes it unattractive to keep participating in the task. As cognitive tests with pigs do not succeed if participation in the test is forced, it may be hard to find an efficient penalty.

The second task designed was a cognitive pig holeboard. This task was applied previously in pigs (Arts et al. 2009). In our slightly modified version of the cognitive holeboard task piglets and pigs from different ages, sizes, gender and lines were willing and able to perform well. As described in chapter 2, the requirements of a broad and reliable cognitive task for pigs are fulfilled by the cognitive holeboard task. After automation of behavioural scoring (chapter 5), the number and accuracy of the parameters measured could even be increased. Performance of pigs exceeded performance of rats (Bouger and van der Staay. 2005), the most frequently tested species in this type of tasks. Pigs acquired the reward-configuration well and rapidly learned a new configuration (reversal learning).

Further comparison of the simultaneous discrimination task (chapter 3) with the cognitive holeboard task for pigs (described and applied in chapter 4, 5 and 6 and appendix I) reveals remarkable differences in motivation to perform the task. Criteria for test development (such as tapping a detailed behavioural analysis and ecologically relevant behaviour) that were not fulfilled in the simultaneous discrimination task were much easier to fulfil with the holeboard task. In the holeboard, the animal is allowed to move freely in the test arena and choices are never forced: the pig can show behaviour that much more resembles natural foraging (described in chapter 2). A maximum of 25% of the choice alternatives (holes) leads to a reward (compared to a fixed 50% in the simultaneous discrimination task) and there is a larger distance to cover between rewards. Therefore, motivation to remember previously rewarded and unrewarded locations might be higher, because it benefits (fast) performance. As the time limit is fairly irrelevant, because in ten minutes all bowls could have been visited many times, it seems that the animals are driven by the naturalness of the task demands and by the rewarding nature of searching for food. Hunger can be excluded as the main drive to perform because the pigs in none of the experiments were food deprived. When the amount of food was not restricted but unevenly spread over the day (⅓ before testing and ⅔ after testing) no performance differences were found in the cognitive holeboard test.

In conclusion, the cognitive holeboard task is a task matching most of the criteria defined in chapter 2. Therefore we decided to continue developing the cognitive holeboard task. Automation and validation steps were taken (chapter 4 and 5) to improve the manageability of the task and the reliability of results and to see whether it yielded robust and replicable results.

Validity of the cognitive holeboard test

Validity of a cognitive holeboard task was assessed by investigating whether it is able to detect within-subject differences after a (pharmacological) intervention and differences between groups that underwent experimental treatments (between-subject effects). To improve the usability of the cognitive holeboard test for studying naturally occurring or experimentally induced cognitive deficits, the effects of husbandry systems on cognitive development, or possible cognition enhancers in pigs, more clarity was desired about the validity of this behavioural test and its ability to discriminate between subtle differences in cognitive performance. Therefore several steps were undertaken:

a) Performance of two groups of piglets with a low and normal-birth-weight was compared;
b) Scoring of behaviour in the test was fully automated;
c) The acquisition of the holeboard task of two different pig lines was compared. Then both lines were treated with an increasing dose of a putative cognitive impairer (pharmacological intervention).

a) Piglets born with a low (LBW) and normal-birth-weight (NBW) were compared in the task. Main findings were that both groups were well motivated and able to learn the task. When switching from the originally learned configuration of baited holes to a new one, the LBW piglets showed to have more difficulty to adapt to the new situation. Their response flexibility was initially lower than that of their NBW siblings. These results were the first indication that the cognitive holeboard task for pigs is able to detect subtle differences between groups and that the task is not too easy or too difficult to acquire.

b) Next, an automation step was taken; the 'holes' were equipped with sensors and all visits were registered by software specifically written for the cognitive pig holeboard ('Experiment control for University Utrecht', Blinq Systems, Delft, The Netherlands). This step solved any reliability problems that may be caused by intra- and inter-observer fluctuations during data collection and is expected to increase replicability of data derived in the same or different labs.

c) A following step in the validation process included a pharmacological intervention with the putative cognition impairer biperiden in two different pig lines that were trained, treated and tested in the cognitive holeboard task. During a preliminary pilot study (see appendix I) it was also attempted to show test validity pharmacologically by treating the pigs with the non-selective muscarinic antagonist scopolamine, a cognition impairer. Because of the difficulty of reliable drug administration and uptake encountered during testing, and the current controversy about scopolamine as the golden standard for inducing memory impairments (as this drug has a wide spectrum of behavioural effects besides inducing cognitive impairment) (Klinkenberg and Blokland. 2011), we decided to continue with the more promising drug biperiden (Klinkenberg and Blokland. 2011) which acts as a selective M1 receptor antagonist.

As during the LBW study (chapter 4), it was again shown that subtle differences between groups could be detected with this test setup: comparing conventional pigs with Göttingen minipigs we found that both lines were well able to learn the configuration of rewarded bowls, but that minipigs learned this at a slightly slower pace. Eventually, the same level of performance was reached by both breeds. Pharmacological intervention using biperiden as putative cognitive impairer in these healthy and unimpaired (mini)pigs induced only marginal effects on both reference and working memory (WM) at very high oral doses. Therefore it was not possible to unambiguously conclude that biperiden transiently impaired spatial memory and that this could be shown with the cognitive pig holeboard. This step in the validation process showed the holeboard to be able to detect within-subject differences after a pharmacological intervention, although several experimental

flaws (such as determining the correct moment to commence treatment during the training phase, the correct dosing regimen, dose and way of administration) should be avoided in future experiments to be able to draw more solid conclusions about the validity of the cognitive holeboard when testing pharmacologically induced cognitive impairments.

The piglet as a translational model for LBW in humans

The reasons for development of the piglet as a translational model animal for LBW in humans (but also in its own species, although this would not be called translational), have been explained in the introduction. This model would be meaningful if it helps to gain insight into the cognitive (dys)functions and other behavioural effects in children born with a prenatal growth restriction. Continuation of studies with this model should be based on the outcome of the evaluation whether this model meets the requirements of face, predictive and construct validity. If the criteria of predictive and construct validity are not met then the relevance of the model is questionable (van der Staay. 2006). Face validity asks if the behavioural or physiological symptoms observed correspond (are phenomenologically similar) to those observed in human patients (McKinney and Bunney. 1969). Predictive validity is successful if the model discriminates between effective and ineffective treatments (Willner. 1986). Construct validity relates to homologous constructs being studied in humans and animals (Willner. 1986). With this approach, it should be determined to what extent the model incorporates the theory as proposed (Sarter. 2006).

Face validity

Emotional and cognitive symptoms

'Cognitive impairment' is a very broad description of the consequences of IUGR on the brain and comprises a range of more specific problems such as a lower brain weight (Cox and Marton. 2009), learning problems and spatial orientation difficulties (Chaudhari et al. 2004, Hack. 2006, Kessenich. 2003) (a more detailed description can be found in the introduction to this thesis). To investigate to what extent the IUGR symptoms of children and pigs are similar, cognitive tests are needed. In general, the tests applied in humans differ from those applied in animals and often involve skills as reading and math. These skills are difficult to compare with the cognitive skills of pigs, but some learning and memory tests can be applied across species. The cognitive pig holeboard is one of these tasks (van der Staay et al. 2012). The cognitive pig holeboard assesses spatial learning and memory: is an animal capable of learning where the rewards are located and how fast does it acquire the task? Because in the holeboard it is possible to distinguish between two types of memory (WM and RM), more specific conclusions can be drawn about what aspects of cognition are affected by the birth weight. By applying reversals from one reward configuration to another, it is possible to investigate the response (in)flexibility of the animals. This (in) flexibility or behavioural inhibition, that is visible if the animal is confronted with an unexpected change in the test environment, is comparable to the finding that IUGR children appear to be less adaptive to changing test conditions: they show difficulties in producing correct strategy solutions (Leitner et al. 2005). Decreased response flexibility is shown in LBW piglets in the cognitive holeboard

test (Gieling et al. 2012). These LBW piglets showed mild but transient deficits: they had difficulty during the initial phase of the reversal, but this difference disappeared with further training. The working memory component of spatial memory was affected, as was the latency to find the first rewarded food bowl. However, in a subsequent study with LBW animals (chapter 6) these results could not be replicated. The main difference between these studies was the moment at which the animals were subjected to the reversal. In the first study the configuration of all animals was switched after 26 training trials. The second study applied a strict learning criterion and all animals were trained for at least 40 trials before the configuration was changed. The different approaches in training could have caused the different results. Alternatively, the LBW and NBW cohorts of animals could have differed too much between experiments. As mentioned before, LBW can have many causes and can originate during all stages of pregnancy. Because a model of naturally occurring animals is studied, these factors could largely influence the homogeneity of the population and are beyond experimental control.

On the other hand, it is questionable to what extent the human LBW or IUGR population can be seen as homogeneous (Lundgren and Tuvemo. 2008). Not all human studies distinguish between whether LBW neonates are born term or preterm (e.g. Breslau et al. 1996, Fattal-Valevski et al. 1999), some add birth weight classes (e.g. extremely LBW, very LBW and LBW) to the study (Hack et al. 1992, Litt et al. 2005), different cut off points for LBW are used, and infants born with major handicaps are excluded (e.g. Breslau et al. 1996, Delgado-Rodriguez et al. 1995). Note that many but not all LBW studies have found implications, or only marginal implications, of LBW for cognitive performance (e.g. Sommerfelt et al. 2000, Theodore et al. 2009).

In an open field and novel object test applied to study emotional reactivity, LBW piglets were found to show a higher number of vocalizations during isolation compared to NBW age-matched siblings (chapter 6). Assuming that the number of vocalizations is an indicator for the level of stress or anxiety in piglets, this study shows comparable results as an epidemiological study showing that very LBW children display an increased level of anxiety (Hayes and Sharif. 2009).

Physical and physiological symptoms

Along with the emotional and cognitive similarity between the human LBW child and the pig model, additional physiological findings that are comparable between LBW humans and pigs could strengthen the face validity of this model. Body weight gain (chapter 3) and final body weight of LBW animals (chapter 3 and 6) was found to be lower compared to NWB animals. This is in line with the human situation, in which sufficient postnatal compensatory growth does not always occur (Garcia Coll et al. 1996, Pryor et al. 1995, Xiong et al. 2005), and the same children that remain atypically small have greater chances of less than optimal cognitive development (Casey. 2008).

The absolute brain and hippocampus weights of LBW piglets were found to be lower compared to those of NBW siblings, while their relative weights were higher (chapter 6). Low hippocampal volumes were found in MRI studies of preterm

LBW children (Isaacs et al. 2000). As the relative weights in LBW piglets were higher, this might suggest a certain degree of brain sparing in the LBW animals, which is comparable to findings in humans suffering from IUGR (Barker. 2004, Cheema et al. 2009, Klaric et al. 2012).

Summarizing, cognitive and emotional symptoms observed in IUGR in humans and in the model that plea for its face validity are decreased response flexibility in a spatial learning and memory task and an increased anxiety level. However, decreased response flexibility in LBW piglets was not replicated. Replicability is an important prerequisite for the validity of the model (van der Staay. 2006). The heterogeneity of the LBW population, in humans as well as in pigs, might be the major factor for these variable findings. Physical symptoms showing overlap between the model and human IUGR are body weight and body weight gain, but also absolute and relative brain and hippocampal weights. It can be concluded that the IUGR piglet model to a certain extent meets the criterion of face validity for specific symptoms, although the naturalness of the model – and therefore the uncontrollability – could cause batches of LBW piglets to show a large variation in phenotypes. We expect that the more and better matching criteria between the human and model situation can be taken into account when including piglets in experiments, the better the face validity of the model will become.

Predictive validity

The predictive validity of this animal model was tested in one extended study administering ALLO to pregnant sows during the last trimester of pregnancy, but has never been tested with a proven effective drug, a cognition enhancer. ALLO is believed to be beneficial in the prevention of brain damage during acute birth asphyxia (Boda et al. 1999, Kaandorp et al. 2010b, Torrance et al. 2009) and during more chronic hypoxia during pregnancy (van Bel et al. 2006). Taking into account that many aspects around effective application of this drug are still unknown, this approach will not reveal a decisive indicator for concluding whether the LBW piglet model has predictive validity.

However so far, one minor indication was found that ALLO is able to differentially influence LBW and NBW piglets. Studying relevant brain plasticity markers in the BDNF (brain derived neurotrophic factor) mediated neuronal plasticity pathway (chapter 7) in ALLO treated LBW and NBW piglets at 5 – 5.5 months of age revealed a trend towards a differential effect of ALLO treatment in the two birth weight groups. Chronic prenatal ALLO treatment appeared to induce a lasting increase of the pCREB/CREB ratio ((phospho-) cyclic AMP–responsive element binding protein ratio (Silva et al. 1998) in dorsal hippocampus tissue of LBW piglets, but to decrease this ratio in NBW animals. If CREB is activated by phosphorylation this can lead to the expression of a wide range of proteins. By phosphorylation pCREB is formed and as this protein is required for a variety of complex forms of memory, including spatial memory (Silva et al. 1998). Therefore, a higher pCREB/CREB ratio may be beneficial for the cognitive performance of LBW animals. The present finding, however, needs to be replicated and corroborated in a larger scale follow-up study before one can conclude that the present animal model has predictive validity for the effects of ALLO.

Further evaluation of the predictive validity of the LBW piglet model should include pharmacologic treatment with an efficient cognition enhancer, although selecting the most appropriate drug, the optimal route of administration and treatment schedule, and the most effective dose would require numerous studies (see, for example de Jongh et al. (2008)). Evaluation could include many aspects as physiological perinatal parameters, learning and memory performance in the cognitive holeboard, emotionality in the open field and novel object test, final body, brain and organ weights. It is possible that not all aspects included clearly show predictive validity. It is important to keep in mind that this is not caused by a flaw in the model *per se*, but that it can also be related to the diagnostic tools used (Vorhees. 1987). That some aspects do and some aspects don't show predictive validity could for example be related to the sensitivity of the tests used to measure the behavioural or physiological outcome (as explained earlier in 'validity of the cognitive holeboard task'), but it may also account for false positive results (Vorhees. 1987).

Construct validity

Assessing the construct validity of animal models of mental disorders is difficult because many questions around their underlying causes and mechanisms remain unknown. The main hypothesis of LBW-related cognitive deficits is that these deficits are induced by placental insufficiency, causing periods of hypoxia and re-oxygenation (Cox and Marton. 2009, Ergaz et al. 2005), which in turn cause damaged brain cells. This hypothesis is proposed for the human as well as the piglet (Cox and Marton. 2009, Blomberg et al. 2010, Ergaz et al. 2005). What is precarious about this hypothesis is that placental insufficiency is seen as the most frequently occurring cause, but many other possibilities cannot be excluded (Cox and Marton. 2009). Within this natural model, causes of LBW related to malnutrition or substance abuse would not occur unless induced, as the sows' environment is strictly controlled. Causes as chromosomal deficits are more difficult to intercept and distinguishing between IUGR caused by chromosomal deficits or placental insufficiency therefore more difficult. As the LBW piglet is an animal that occurs naturally, only the environmental conditions of the sow and piglets (after birth) can strictly be controlled. In case of natural delivery, perinatal complications cannot be foreseen, but events such as birth asphyxia could be monitored per piglet. This could be sensible to ensure that piglets affected by perinatal adverse events can be excluded from purely IUGR affected piglets.

Some pig studies have focused on the difference between symmetrical and asymmetrical growth restriction (e.g. defined as the ponderal index: birth weight (kg)/crown-rump length (m^3), (Baxter et al. 2009)). The asymmetrically- growth piglets would have suffered from placental insufficiency, while the growth restriction of the piglets showing symmetrical growth would have started during early pregnancy and is more likely caused by a chromosomal deficit. (Klaric et al. 2012) Not only selecting for birth weight but also including the ratio between head and body size could probably refine the model and restrict the animals included in a study to animals that most probably show IUGR caused by placental insufficiency.

Construct validity of this LBW piglet model seems to be met, because the same theories likely explain the cognitive deficits found in LBW newborns in both species. Not all factors involved in the prenatal development of the growth-restricted foetus can be controlled in a model based on a naturally occurring deficit. This should be taken into account.

A practical application of the LBW model: prenatal treatment with allopurinol

A pharmacological approach to reduce the negative cognitive effects of brain damage in IUGR piglets has been evaluated in this thesis. This meant an application of the LBW piglet model in the cognitive holeboard task: foetal piglets underwent chronic treatment with ALLO to reduce the damage possibly caused by hypoxia and re-oxygenation related to IUGR.

As ALLO is known as a pharmacological compound that reduces hypoxic damage around acute birth asphyxia in humans and piglets (Derks et al. 2010, Marro et al. 2006, van Bel et al. 1998) and it was hypothesised that comparable results could be found in growth restricted organisms, its safety and efficacy for preventing deficits in the cognitive performance of LBW animals after a prolonged treatment period were assessed. As a first step, the correct oral dosing regimen was determined experimentally.

Therapeutic range and dose

Although ALLO has often been reported to be a treatment for human and animal neonates that suffered from hypoxic insults, no consistent information regarding the dosage, administrative regimen, duration or timing is available (Masaoka et al. 2005). Torrance and colleagues (2009) suggest that neuroprotection starts with plasma levels above 2 $\mu g.ml^{-1}$ for ALLO and for its active metabolite oxypurinol (OXY) above 4 $\mu g.ml^{-1}$. To scavenge damaging free radicals, higher doses might be needed (Pacher et al. 2006). In case of treatment during or even after the hypoxic period a high dose could be beneficial, however, this might not be needed if ALLO is applied as a preventive therapy where the formation of free radicals should be chronically reduced. 'High' dosages (30-40 $mg.kg^{-1}$) have been administered in human (van Bel et al. 1998) and pig (Boda et al. 1999) experiments. These dosages are considered as 'high' in relation to the doses prescribed in case of gout and to reduce the level of uric acid in the blood (e.g. 7-20 $mg.kg^{-1}day^{-1}$ in dogs (Dallwig. 2010)). In case of gout, ALLO is administered chronically, while in most experimental studies the drug was only given once or for a short time period. The incidence of adverse effects may be different, depending on whether the compound is administered chronically or as one single administration, although it is said to be a relatively safe drug (Dallwig. 2010). Above a concentration of 13.6 $\mu g.ml^{-1}$, toxic side effects on skin, white blood count and liver enzymes were frequently detected in human neonates (van Bel et al. 1998).

To study the preventive effects of prolonged ALLO treatment on IUGR foetuses, as described in chapter 6, ALLO had to be administered during the last trimester of pregnancy. To apply a treatment that was also relevant for the clinic, ALLO

was administered orally. Pharmacokinetics of single oral dosing was studied in four pregnant sows and their piglets. The results were extrapolated to calculate an optimal dose to reach steady state with daily administration and accurate plasma levels for ALLO and/or OXY according to the range of neuroprotection suggested by Torrance et al. (2009). Finally, 15 $mg.kg^{-1}$ was defined as a suitable dose to reach a steady state for ALLO. OXY levels were low in every measurement in the piglets. Therefore, it is likely that OXY molecules are not able to cross the placenta and it may be that ALLO cannot (or only in minimal amounts) be converted to OXY in the liver of very young piglets (van Dijk et al. 2008). Therefore, it is unlikely that OXY has a significant therapeutic effect on the pig foetus.

To control for accumulation of ALLO or OXY in the sows, plasma levels were analysed during a preceding study with two sows (both sows were sampled on day three and ten of treatment) and no accumulation occurred. Their piglets were sampled after birth through caesarean section. A selection of piglets (n=8) was sampled and the average plasma levels were found to be 3.23 $\mu g.ml^{-1}$ for ALLO (only one piglet showed a plasma level <2 $\mu g.ml^{-1}$) and below the detection limit (0.01 $\mu g.ml^{-1}$) for OXY in all piglets. From every sow, one piglet was sampled after five hours and another one after 24 hours. ALLO levels were still clearly above 2 $\mu g.ml^{-1}$ after five hours, but not after 24. No signs of toxicity or other side effects were noticed in any of the sows or piglets during or after the treatment period. Based on this pharmacokinetic information a subsequent study was performed, using six sows, which were chronically treated with ALLO and another six untreated sows that served as controls.

Effects of ALLO on the cognitive performance of LBW piglets

Chronic ALLO treatment of the sow during the last 30 (±2) days of pregnancy did not lead to a better performance in LBW piglets in the cognitive holeboard. Also, as described earlier in 'face validity', we could not replicate the effects of LBW on holeboard performance found in a previous study (chapter 4). With no learning and memory differences between LBW and NBW piglets, it is not surprising that no positive effects of ALLO treatment on cognitive performance were found (expect if ALLO would have acted as a 'cognition enhancer', in which case all ALLO treated LBW and NBW piglets should have benefited). Therefore, to draw solid conclusions about the effects of ALLO on the cognitive performance of LBW piglets in the cognitive holeboard test, it should be clarified first further whether 1) the LBW piglets selected showed enough similarity with the group of children considered LBW 2) the difficulty of the cognitive holeboard task for pigs was set at the optimal level (a suboptimal difficulty level could lead to false negative results). Additionally, as mentioned in chapter 6, the power of the experiment should be sufficient: because the sow was the experimental unit in this experiment, the number of units was lower than in the experiment described in chapter 4.

1) Piglets versus human neonates

The LBW piglet model uses animals with naturally occurring symptoms and before parturition it is difficult to obtain clarity about the number of LBW piglets a sow is carrying and the level of their growth restriction. As it is possible that no or only a few LBW piglets are found in a litter, this means that for both the treatment and

the control group more animals would be needed to ensure that a sufficiently large number of animals will be available to draw statistically reliable conclusions.

The LBW piglets are born and reared under farm conditions and there is normally no special care for these LBW neonates. In particular, the most growth restricted and vulnerable LBW animals are at high risk for neonatal complications (Gondret et al. 2005, Milligan et al. 2002, O'Reilly et al. 2006), while in a hospital a human neonate is observed frequently or is even placed in a NICU (neonatal intensive care unit) when necessary. LBW piglets under farm conditions might weaken and eventually die. Their chances of survival would be much higher if they would receive appropriate care. A template for a piglet NICU (PNICU) exists, especially designed for translational research (PNICU; Lennon et al. 2011). An additional reason to care after the LBW piglets in a different way is that it is known that the development of the neonate depends on the environmental conditions it is brought up in (Breslau and Chilcoat. 2000, Gardner et al. 2003). Therefore, the neonatal environments (especially the level of care and hygiene) should be comparable between the human situation and the translational model. These adjustments could improve comparability between the model and the real world situation. Groups of selected LBW piglets would be less biased (due to a higher chance to survive) if the level of care would be comparable to that of the human neonate. It should, however, be taken into account that the pig is a highly social species and therefore a prolonged period of total social isolation from its siblings and the sow, as in a traditional incubator, might be detrimental for its emotional and cognitive development. This likely would affect the results of behavioural testing. The PNICU environment should as far as possible be adapted to the needs of this species.

2) Optimal difficulty level of the cognitive pig holeboard

Defining the optimal level of difficulty of a learning and memory task is important in order to avoid false negative results (chapter 4). The groups to compare should both be able to acquire the task (i.e. perform above chance level), but the maximum level of performance possible should be high enough to show performance differences (if the groups actually differ from each other). For the cognitive holeboard task the most appropriate level of difficulty for healthy, unimpaired pigs has not yet been determined. The task becomes more difficult if a configuration would consist of more than four rewarded holes out of 16. On the other hand, rewarding too many bowls could decrease the motivation of an animal to perform errorless (as the chance to find a reward by chance eventually increases). Additional research into the cognitive holeboard task for pigs could try to optimize the difficulty level by increasing the number of places were bait can be found. Another alternative could consist of changing the rules of the task. For example, reversals could be applied within a session or between sessions. This has been shown to improve the difficulty level of a learning task for pigs in a study by Hagl et al. (2005).

To explore whether the difficulty level of four rewarded and 12 unrewarded bowls in the holeboard was adequate, a measure was included that could provide some additional insight (chapter 6). The number of errors per reward (EpR) shows how

many errors an animal made before it found reward one, between reward one and reward two, etc. When examining RM across training sessions most animals reach a ceiling level of performance. At this point, the measure EpR may provide a descriptive indicator for how difficult the animal perceives the task; is the number of errors between finding rewards similar or does it (suddenly) increase with the number of rewards already found? If the number of errors between finding rewards stays relatively stable and low it shows that the animals' memory load is not increasing. If the number of errors suddenly increases it is likely that memory load is increasing or executive-attention to fulfil the task correctly is declining. 'Slow' performers have to retain the information stored in WM for longer and the list to retain is longer (van der Staay et al. 2012). If the task has a difficulty level within the optimal range, any group differences that are present could show up with this measure. For example, LBW piglets might show a loss of executive-attention earlier during a trial compared to NBW animals. This was not shown during the ALLO experiment by the measure EpR, but it would be an interesting parameter to include in future holeboard studies with LBW animals or other treatment groups.

Effects of ALLO on specific brain plasticity markers

Although chronic ALLO treatment of the sow during the last 30 (±2) days of pregnancy did not lead to a better performance of LBW piglets in the cognitive holeboard, some relevant effects on brain plasticity markers in the BDNF mediated neuronal plasticity pathway were found (chapter 7). Increased mBDNF (mature brain-derived neurotrophic factor) and PSD95 (postsynaptic density protein 95) protein levels were found in treated (LBW and NBW animals) compared to controls. As discussed under 'predictive validity' also a marginal birth weight*treatment interaction for pCREB/CREB was revealed by Western-Blot analysis. A study by Wang and Xu (2007) assessing the effects of malnutrition during pregnancy in rats found that BDNF levels in the hippocampus were significantly lower in pups that suffered from protein malnutrition compared to controls. These animals also showed impaired spatial memory performance, an effect that partially may have been caused by the lowered BDNF levels in the brains of animals that underwent malnutrition. In our study, there is a discrepancy between a lack of effects on cognitive performance in the holeboard task after ALLO treatment and an increase in memory-related brain plasticity markers. The increased plasticity markers could be seen as indicators of efficiency of chronic prenatal ALLO treatment in LBW animals. However, as pCREB/CREB ratio seemed to increase (which could be beneficial) in LBW, but decrease in NBW animals, one should be cautious to draw conclusions. A plausible reason for this discrepancy between brain and behavioural measures could be that subtle changes in the brain do not necessarily induce behavioural effects. These changes may have been too small to be of physiological significance and to affect cognition. The discrepant findings could also be due to differences in the sensitivity of the biochemical and behavioural assays used.

Conclusions and recommendations for future research

To study the cognitive performance of piglets suffering from intra-uterine growth restriction and to investigate whether chronic ALLO treatment is safe and efficient in preventing possible adverse consequences of IUGR on cognition, using piglets

as subjects, was the main aim of this thesis. To address this aim more specific questions had to be studied. The main findings based on these questions described in chapter 1 are as follows:

The cognitive holeboard test

- A cognitive test for pigs should match the species-specific behaviour and capabilities, as ability and motivation to perform are key factors involved.
- The cognitive holeboard task is a spatial learning and memory task that seems to fulfil most of the general criteria defined for behavioural tasks for pigs (Gieling et al. 2011). Most important the task is acquirable by healthy and unimpaired animals; to a large extend stress-free; taps ecologically relevant behaviours; allows for a detailed behavioural analysis; and scores behaviour in a fully automated way.
- Automation and validation steps of the cognitive holeboard task clearly improved the reliability of testing.
- The most optimal difficulty level for the cognitive holeboard task to be able to detect subtle differences between treatment-conditions must still be determined.

The LBW piglet model

- LBW was found to affect the response flexibility of LBW animals after presenting a reversal in the cognitive holeboard task (chapter 4). However, these results could not be replicated in a follow-up experiment (chapter 6).
- The model is considered to be suitable for further development and practical application, when the LBW piglet model is evaluated using the face, predictive and constructive validity framework.
- The naturalness of the model increases the validity but at the same time is a drawback as it limits controllability of putative relevant variables and increases heterogeneity of experimental and control groups.
- The translatability of the LBW piglet model could be strengthened if the postnatal care of piglets would rise to a level comparable to neonatal care in developed human societies.
- The LBW piglet model could enhance our knowledge about the possible implications of growth restriction in piglets. These implications could be discussed and lead to improved welfare regulations with respect to these vulnerable farm animals.

Allopurinol as a therapy to prevent brain damage in IUGR neonates

- Sows and piglets chronically treated with ALLO during the last trimester of pregnancy did not show any detrimental effects during pregnancy, the parturition process, piglet growth and with respect to general health status if compared to untreated controls.
- A beneficial effect of ALLO treatment on cognitive performance could not be demonstrated: chronic prenatal treatment with ALLO did not lead to a better performance of LBW piglets in the cognitive holeboard task. This may be due to the fact that the behaviour of untreated LBW piglets in the ALLO experiment did not show any cognitive deficits.

- Long-lasting increased mBDNF and PSD95 protein levels in the dorsal hippocampus of ALLO treated pigs are a promising indicator for the efficacy of chronic prenatal ALLO treatment. Both proteins are part of the BDNF mediated neuronal plasticity pathway that is involved in spatial memory performance.

Conclusions

The cognitive pig holeboard is found to be a reliable behavioural task for measuring several components of spatial learning and memory of various pig breeds from the age of weaning onwards. Automation of the task increased its reliability, and the proven sensitivity to experimental manipulations (e.g. LBW vs. NBW, commercial pigs vs. minipigs, pharmacological treatment with the putative cognitive impairer biperiden) supports the notion that this test is valid.

Piglets born with a prenatal growth restriction, i.e. LBW piglets, are a promising model animal for mimicking IUGR in humans. This model shows an appropriate level of face, predictive, and construct validity and is therefore suited for further development. However, this model needs further refinement and validation.

The cognitive capacities of LBW piglets can be tested in the cognitive pig holeboard. Results so far have shown that LBW piglets are less flexible after presenting a reversal task. A safety and efficacy study was applied in which the LBW piglet model was tested in the cognitive holeboard after chronic prenatal treatment with ALLO. This study did not replicate the cognitive performance differences between LBW and NBW piglets found previously. Also, for cognitive performance there was no difference between ALLO treated and control piglets. Both groups of ALLO treated animals were found to have increased mBDNF and PS95 protein levels, which suggests that ALLO could positively influence development of the hippocampus of LBW animals. Maternal daily administration of 15 $mg.kg^{-1}$ ALLO during the last trimester of pregnancy was found to be safe for the sows as well as their piglets and didn't influence the parturition process. Therefore it is suggested to continue with ALLO studies in the LBW piglet model to assess its neuroprotective potential. Refinement, e.g. by intensifying care of the neonate piglets, will increase the comparability to the human situation and may further improve validity of the model.

Development and validation of the automated cognitive pig holeboard has attributed to the increasing demand for reliable cognitive tasks for this species. With the application of the naturally occurring LBW piglet in the cognitive holeboard task this piglet model could be developed further for its use in translational studies and to assess the safety and efficacy of putative (prophylactic) treatments. Additionally, the application of the LBW model in the cognitive holeboard task might yield new insights into the cognitive effects of IUGR that benefit the welfare of the farmed pig itself.

Appendix 1

Effects of the cholinergic cognition impairer scopolamine on spatial memory in a holeboard task in conventional pigs

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Introduction

Central cholinergic neurotransmission appears to be involved in spatial learning and memory processes (Deiana et al. 2011). Scopolamine (a non-selective muscarinic receptor antagonist) is often employed as the gold standard for pharmacologically inducing memory impairments in healthy humans and animals (Klinkenberg and Blokland. 2010, Klinkenberg and Blokland. 2011). The substance is known to be able to induce Alzheimer-like cognitive deficits on working (WM) and reference memory (RM) (Bouger and van der Staay. 2005), for example in rats. It was shown that administration of scopolamine in well trained rats in a variant of the cognitive holeboard task, the conefield, transiently impaired WM and RM. This effect could be induced multiple times, i.e. scopolamine repeatedly acted as cognition impairers in the same animals. (Bouger and van der Staay. 2005)

We expected that pigs trained in the cognitive holeboard task (see chapter four) subjected to scopolamine in different doses would show dose dependent cognitive deficits.

Materials and methods

Ethical note: The experiments were reviewed and approved by the local ethics committee (DEC, dier experimenten commissie), and were conducted in accordance with the recommendations of the EU directive 86/609/EEC. All efforts were made to minimize the number of animals used and to avoid suffering.

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Animals: Eight experiment-experienced female pigs [(Terra x Finish landrace) x Duroc] bred and raised at the farm of Utrecht University were used during this experiment. The animals were raised under conventional Dutch commercial pig housing conditions. Before the onset of this experiment they were trained in a simultaneous visual discrimination task for pigs. When holeboard training started, animals were eight months old.

Housing: After weaning, at four weeks of age, pigs were moved to the experimental unit. The animals were group-housed in a pen (4m X 5m) in a large naturally ventilated and lighted stable. Minimal and maximal temperatures in the stable were registered daily (3°C - 31°C). The pen contained a covered piglet nest and straw bedding on the concrete floor. During the experiment the animals were fed ⅓ of the daily food amount approximately one hour before the start of behavioural testing (around 08:00 AM) and the remaining ⅔ was fed after testing, around 04.00 PM. Water was provided ad libitum. A radio played between 07.00 AM and 07.00 PM to mask noises of the daily farm routine.

Testing room: The testing room was located adjacent to the housing room, and could be reached via a (straw enriched) waiting area next to the testing apparatus by walking down a corridor. Animals were brought to the waiting area together and tested individually in a randomised order. During testing a radio played continuously. Individuals entering the testing apparatus were still able to hear and smell their pen mates. After testing, an animal was sent to the corridor between the housing and testing compartment until all animals were tested. Then, the entire group of animals was returned to the home pen.

Apparatus: The apparatus, a cognitive pig holeboard (described in more detail in chapter four), consisted of a square arena with a 4x4 matrix of food bowls. The holeboard had four entries (one on each side) which were operated from outside. By voluntarily walking down a small corridor surrounding the entire arena, the animals found the opened door and entered the holeboard. Sixteen food bowls were evenly divided over the arena and each bowl was covered with a synthetic ball. Animals lifted this ball with the snout within a frame on top of the food bowl; this action made the reward available for consumption. The ball rolled back on the bowl as soon as the animal retracted its snout.

Habituation and training: The experiment lasted for a total of seven weeks. Animals were already well habituated to each other, their housing facilities and experimenters. They had already learned to lift a ball to find bait. Habituation to the holeboard took four days. During habituation the pigs were allowed in the holeboard (twice daily) to eat a reward (M&M chocolate) from every food bowl. The animals were trained to find their own configuration of rewarded holes (four out of 16 holes were baited). Four different configurations were used. The entrance door was randomly chosen for every trial. Each pig was trained on a particular configuration for two massed trials a day during 14 weekdays (28 trials in total). The first seven sessions were considered as the learning (training) phase of this experiment.

Scopolamine treatment: Scopolamine (Scopolaminehydrobromide.3aq, BUFA, IJsselstein, the Netherlands) was applied in increasing doses (0.01, 0.3 and 1 mg.kg-1 body weight). To determine the correct dosage, pigs were weighed on the day before treatment. As a control treatment saline (in the same volume as the lowest scopolamine volume needed per kg) was injected in the same way as was scopolamine.

On the first day of treatment (treatments were randomized over animals and equally distributed over treatment days) 0.10 ml.kg^{-1} body weight of scopolamine diluted in saline was injected subcutaneously. Because during the 1st treatment day administering scopolamine or its vehicle in this injection volume behind the ear was found to be difficult, for the following treatment days scopolamine was diluted in smaller volumes of saline (0.3 mg.ml^{-1} for 0.01 mg.kg^{-1}, 10 mg.ml^{-1} for 0.03 mg.kg^{-1}, and 30 mg.ml^{-1}, for 1 mg.kg^{-1} body weight). In this way injection volumes were kept as low as possible to inject easier and faster. Injection of the highest dose was unsuccessful in pig number seven.

Testing: After the training period a two week drug testing period started (Fig 1.). On the (week)day preceding and following a scopolamine treatment (four in total) pigs were trained under drug free conditions. During this period animals were tested with the same configuration of baited holes as during the training phase. Animals were tested for two trials a day in close succession. The wash out period between treatment days was at least one day. On this day (if a weekday) normal training sessions were conducted.

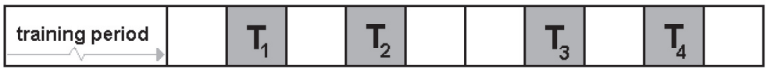

Fig. 1. Timeline of testing the effects of scopolamine. Training period lasted for 14 weekdays and animals received two massed trials per day (28 trials in total). Then a ten day testing period followed. Animals were only tested during weekdays. On treatment days (T1-T4) and days preceding and following a treatment day two massed trials were scheduled per animal.

Data recording: Food bowls (sensor) and balls (magnet) were assembled with magnetic sensors: as soon as the ball was lifted from the bowl, the sensor was activated. This event was registered via an interface, controlled by the software ANY-maze (Stoelting Co., Wood Dale, U.S.A). As soon as the ball fell back on the bowl, the sensor was deactivated. With ANY-maze software trials could be operated (start, stop) and sensor data was collected. Recording and scoring started when a pig entered the arena with both forelegs. A trial was terminated when a piglet had found and consumed all four food rewards or when 600s had elapsed, whichever event occurred first. For the first 13 training sessions unrewarded visits to bowls were scored manually. Rewarded visits (and revisits) were scored with the above mentioned system. After training sessions 13 all visits were scored with this system.

Statistical analysis: The measures Working memory (WM), Reference memory (RM), Trial duration (TD), Inter-visit-intervals (IVI) and Latency to first (rewarded) hole visit were calculated and analyzed statistically.

Working memory, a measure expressing the percentage of all visits to the baited set of holes that yielded a food reward was calculated as: (number of rewarded visits)/(number of visits and revisits to the baited set of holes).

Reference memory, a measure expressing the number of visits to the baited set of holes as a percentage of the total number of visits to all the holes was calculated as: (number of visits and revisits to the baited set of holes)/(number of visits and revisits to all holes).

Trial duration, was the time (s) between entering the holeboard and finding the last reward. If the piglet did not find all rewards, this measure was assigned the maximum trial duration (600s).

Inter-visit interval, or the time (s) per hole visit, was the average time between two successive hole visits. This measure was calculated as the time (s) between the first and the last hole visit divided by (number of hole visits – 1).

Latency to gaining first reward, was the time (s) between entering the holeboard and finding the first food reward.

Acquisition of the holeboard task: For each of these measures block means of four trials per block were calculated. Learning was analysed by an analyses of variance (ANOVA) with the within subject (repeated measures) factor 'blocks'.

Effects of scopolamine: The effects of the treatment on WM, RM and TD were analysed per treatment dose [0.00 (i.e. control), 0.01, 0.3, 1 mg.kg^{-1} scopolamine] per block mean (one session of two trials) by a repeated measures ANOVA with the within subjects factor 'block'.

Analysis responders vs. non-responders

Contrary to the expectations, ANOVA did not confirm effects of scopolamine treatment. Therefore, we examined the WM and RM performance of each individual pig after administration of scopolamine. Based on visual inspection of the performance of each individual pig per dose, pigs were classified as either 'responder' or 'non-responder'. Responders were all pigs that appeared to perform worse during scopolamine sessions, in particular after the highest dose of scopolamine, than in their corresponding vehicle control session. Three pigs were classified as responders; the other five pigs were non-responders.

The effects of increasing doses of scopolamine on WM and RM performance of the two groups of pigs were analysed with a repeated measures ANOVA with the between subjects factor 'responder' (responders vs. non-responders) and the within subjects factor 'block'. Finally, a one-sample t-test were performed to assess whether the delta's [mean saline treatment – mean highest scopolamine dose (1 mg.kg^{-1})] of responders and non-responders deviated from zero.

Results

Training

The WM performance did not improve in the course of training (seven blocks, 28 trials in total) ($F_{6,42}$ = 0.77, p = 0.6002). The initial WM performance level, however, already was very high (WM in first block of four trials: 0.76). By contrast, RM performance showed an increase over the seven trials blocks ($F_{6,42}$ = 12.59, p < 0.0001), accompanied by a decrease in TD ($F_{6,42}$ = 3.96, p = 0.0032), latency till finding the 1st reward ($F_{6,42}$ = 5.46, p = 0.0003) and IVI ($F_{6,42}$ = 3.42, p = 0.0077). These findings are shown in Fig. 2.

Scopolamine treatment

By visualising the data the animals were classified as responders (n=3) and non-responders (n=5). This is shown in Fig. 3. A *t*-test (Satterthwaite for unequal variances) per treatment dose showed no differences between the group means of responders and non-responders after administration of saline or the two lower doses of scopolamine (0.01 and 0.3 mg.kg^{-1}), but the highest dose of 1 mg.kg^{-1} scopolamine affected WM ($t_{4.66}$ 8.80, p = 0.0004). A repeated measures analysis across the saline and scopolamine treatment sessions (with scopolamine in increasing doses) for WM showed that the slopes for responders and non-responders differed ($F_{1,5}$ = 6.68, p = 0.0491). Comparing the delta's (mean saline – mean treatment) of responders (n = 3) and non-responders (n=4, as animal 7 was excluded from this analysis) with a *t*-test (Satterthwaite for

unequal variances), for treatment 1 mg.kg^{-1} a significant difference was found between the two groups ($t_{4.88} = -4.92$, $p = 0.0047$).

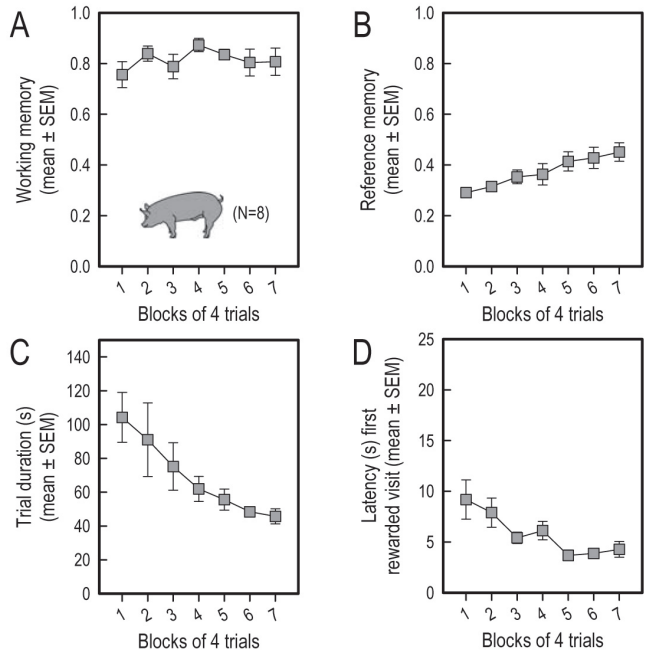

Fig. 2. Learning of a spatial holeboard discrimination task by conventional pigs. The working memory performance (panel A) and the reference memory performance (panel B), the trial duration (panel C) and the latency to first rewarded hole visit (panel D) are depicted as means and standard errors of the mean (SEM) of 7 successive blocks of 4 trials each.

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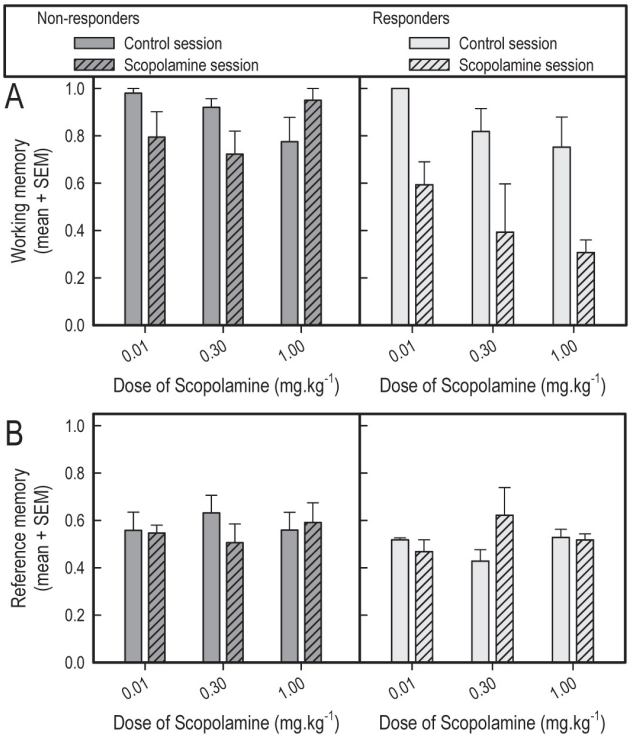

Fig. 3. Effects of oral administration of increasing doses of Scopolamine on working memory (A) and reference memory performance (B) of conventional pigs. The means ± SEM of the drug-free day preceding treatment and of the day of Scopolamine treatment are depicted.

Discussion and conclusion

Only based on visual inspection of the performance of individual pigs after scopolamine treatment, animals were *a posteriori* categorized as responders or non-responders. This yielded a group of three responders and five non responders. The number of animals per group available for statistical comparison between these two groups may not have been sensitive enough to detect effects of the lower doses of scopolamine on WM.

Correct administration of scopolamine (subcutaneously behind the ear) was difficult. The age and weight of the animals (all > 80 kg) might have led to an increased fat deposit at the site of administration. This was suspected as emptying the syringe was very slow in many of the animals. Injecting the substance in a fat deposit could lead to a delayed uptake in the bloodstream compared to proper subcutaneous administration and could therefore have caused lower plasma levels (differing per animal) not leading to the expected cognitive effects. Additionally, injecting itself could have been stress-inducing. Stress is known to affect cognitive performance (Schwabe et al. 2012).

In the 'responder' group, a dose-dependent effect on WM was found; WM performance decreased as the dose of scopolamine increased. This is in line with previous findings in rhesus monkeys (Taffe et al. 1999) and rats (Bouger and van der Staay. 2005). As the number of animals and measurements in this study is very limited these findings can only be taken as a cautious indication that scopolamine, subcutaneously administered behind the ear, caused a dose-dependent WM deficit.

Appendix 2
The cognitive pig holeboard visualised

Drawings: Y. van der Staay

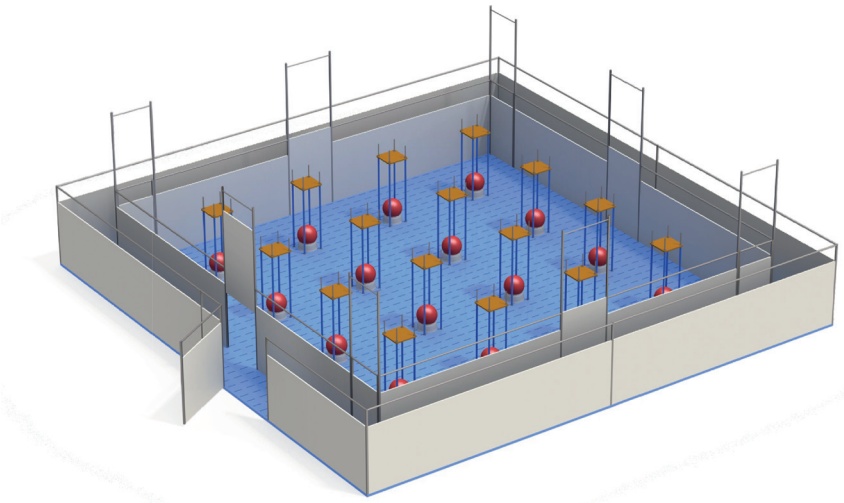

Fig. 1. Upper view of the pig holeboard apparatus. A square arena with slatted floor and 16 covered food-bowls present ('holes'), surrounded by a corridor. One entry door to the corridor and four sliding entry doors to the arena are present.

Fig. 2. Constructional details of a hole of the pig holeboard apparatus. The food bowl is equipped with a magnet-sensor. Below a false bottom M&M chocolates are placed to prevent pigs from detecting rewards based on their smell. The actual M&M chocolate reward, if present, is placed on top of this false bottom. If the ball is lifted from the bowl with the snout, the signal between sensor and magnet is broken and this is registered as a 'hole visit'. After retracting the head, the ball falls back in place.

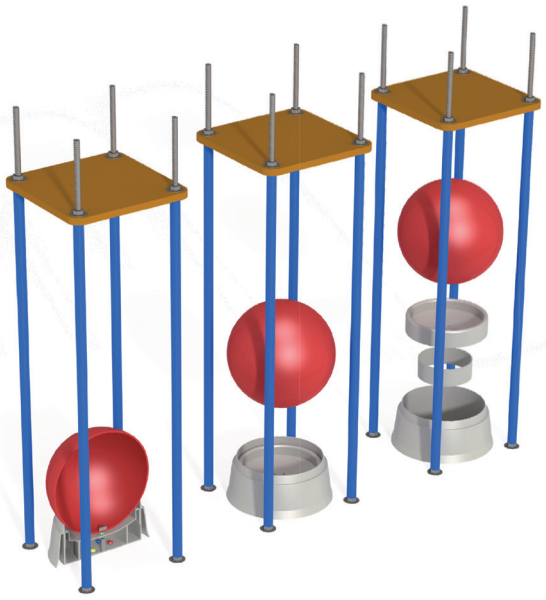

Fig. 3. Constructional details of the holes of the pig holeboard apparatus. Left panel: cross section of a “hole”, covered by a ball, containing a magnet. Center panel: Ball lifted, exposing the food trough. Right panel: exploded view of a ‘hole’.

Appendix 3

A measure to investigate task-difficulty of the cognitive (pig) holeboard: the number of errors per reward

Elise Gieling
M.C. van Herel

Introduction

The cognitive holeboard task was originally developed to test rats; although versions for mice and other species are also available. Several well-validated measures exist to assess learning and short- and long term memory in the cognitive holeboard task, in particular for rat studies (van der Staay et al. 2012). The optimal task difficulty for, and ceiling level of performance of rats became apparent over years of testing. As the cognitive pig holeboard task is a relatively new task and only few results are available (Arts et al. 2009, Gieling et al. 2012, Manton. 2010), optimal task difficulty has not yet crystallised. Determining the optimal task difficulty is important for future valid and explicable results (Gieling et al. 2011). Using data from our automated cognitive pig holeboard experiments (see chapter 5 and 6) we worked out a measure that provides insight in the number of errors a pig makes between finding successive rewards.

This measure can be used in a descriptive way to interpret the difficulty level of the test per group or animal tested. Usually four out of the sixteen sites where a pig can look for bait are rewarded. The more sites (food bowls) an animal visits during a trial, the more within-trial information it has to store in its working memory, and consequently, memory load increases. If none or only a few errors (e.g. visits to unrewarded or revisits to rewarded bowls) are made between visits to rewarded bowls, memory load stays limited. If the number of errors increases when later rewards are still to be found, probably memory load is increasing or executive-attention to fulfil the task correctly is declining or eventually lost. The latter could also be interesting in case of testing LBW piglets, as it has been hypothesized that cognitive impairment in IUGR children is due to an executive-attention deficit (Geva et al. 2006).

Methods

Description of the Measure: The measure 'Errors per reward' (EpR) shows the number of errors (unrewarded visits or revisits to rewarded sites) an animal makes before finding reward 1-4 (in case of a configuration of 4 out of 16 rewarded food bowls, as is commonly applied in rodent studies and has also been adopted in our pig experiments). The errors are counted per reward found and are not accumulated over rewards (see Fig. 1).

Data collection: The data used for calculation of this measure were derived from a cognitive pig holeboard (manufacturer Ossendrijver BV, Achterveld, the Netherlands, for details see appendix II) experiment. Automated data collection was accomplished with software especially designed for the cognitive pig holeboard ("Exp. Control for Utrecht University", Blinq systems, Delft, the Netherlands) and processed into a Microsoft Excel data file. Data necessary for calculation of this measure were the number of errors an animal makes between every first visit to one of the four rewarded food bowls (depicted in Fig. 1) in random order.

The better an animal or treatment group performs, i.e. the less errors are made, the lower (i.e. closer to zero) and the more horizontal an EpR line should become. Naturally this is only the case when task difficulty allows for errorless performance

and when training has progressed far enough. When the task is so complicated that an animal does not reach errorless performance (or a WM and RM ratio measure of 1), even after extended training, the graph representing EpR at the end of the training period, shows from what reward on an animal or group of animals starts to make remarkably more errors compared to finding previous rewards. This is depicted in Fig. 2.

Representation of results: Results can be represented as shown in Fig. 2. If groups of animals are compared, normally group means (± SEM) are depicted, however presenting the median (± SEM or a cloud of the actual performance per individual) could in this case be more descriptive. A mean can be influenced by a very good or very bad performance of a single pig. The median provides insight in the performance of the most intermediate pig within the group. An example is show in Fig. 2C.

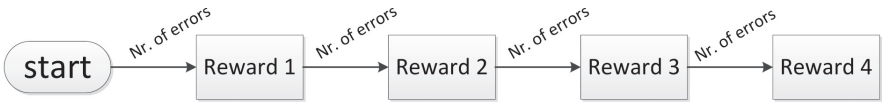

Fig. 1. Graphic representation of the number of errors per reward (EpR). An error is every re-visit to 1) a rewarded food bowl during the same trial or 2) every visit to a food bowl of the never rewarded set of bowls. When an animal revisits the same unrewarded or previously rewarded bowl within ten seconds this is not counted as an error, except for when another bowl is visited in between. The number of errors is not accumulated over rewards

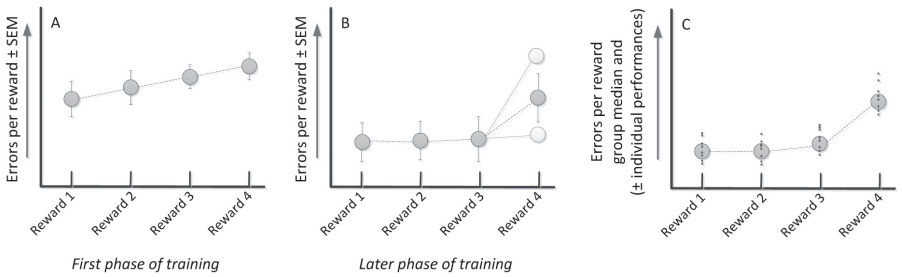

Fig. 2. Representation of hypothetic results of the number of errors per reward (EpR) during two different stages of training. During a later phase of training it can be observed if the animal (or group of animals) is (are) able to acquire an errorless level of performance or increases the numbers of errors made from a specific number of rewards found onwards (depicted in panel B. Different possibilities are depicted). In panel C, instead of group means, group medians per reward found are shown and complemented with the individual performances per animal (represented as individual points).

Statistical analysis: Holeboard data is usually analysed per trial block (where a block represents, for example, the mean of four successive trials) to reduce variance. If one is interested in individual performance of animals or groups at a specific time-point during training, EpR can be compared within such a block: does it increase or stay level over the rewards found? To investigate performance of animals or groups over time (i.e. a training period), one can compare the EpR slope per block within an animal or group using a repeated measures ANOVA with EpRs as first, and trial block as second repeated measures factor.

Conclusion: The measure EpR can easily be derived from data collected in the automated pig holeboard. It is useful to determine the level of difficulty of a configuration of rewarded bowls, which is important to determine whether the task is not too easy or difficult for the animals tested.

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Nederlandse samenvatting

Cognitieve implicaties van een laag geboortegewicht

Baby's die worden geboren met een laag geboortegewicht (LGG) hebben een verhoogde mortaliteit en morbiditeit. Daarnaast lopen zij op het psychische en cognitieve vlak onder andere een verhoogd risico op een suboptimale ontwikkeling, depressies, problemen met leren, ruimtelijk inzicht en aandachtsproblematiek. Gebaseerd op statistieken van de Wereld Gezondheidsorganisatie (World Health Organisation; WHO) worden er wereldwijd 30 miljoen (23.8%) baby's per jaar geboren met een laag geboortegewicht. Dit cijfer is lager in ontwikkelde, maar beduidend hoger in minder ontwikkelde landen. Omdat voornamelijk in de ontwikkelde landen de perinatale gezondheidszorg sterk verbeterd is, overleven steeds meer baby's met een laag geboortegewicht de eerste kritieke periode na de geboorte. Niet alleen aan het overleven, maar ook aan de kwaliteit van leven wordt de laatste jaren steeds meer waarde gehecht.

Biggen met een laag geboortegewicht

Ook biggen worden zeer regelmatig geboren met een laag geboortegewicht. Door het selecteren van fokzeugen welke grote tomen werpen, worden er binnen de conventionele vleesvarkenshouderij veel LGG biggen geboren. Slechts een deel van deze biggen overleeft de kritieke neonatale periode. Of deze biggen net als humane neonaten een cognitieve achterstand ontwikkeld hebben ten opzichte van leeftijdsgenoten met een gemiddeld geboortegewicht is onbekend.

Cognitieve studies bij het varken

De laatste jaren worden varkens en minivarkens steeds geschikter geacht voor gebruik in cognitief onderzoek. De cognitieve eigenschappen van het varken worden onderzocht ten behoeve van dierenwelzijn maar voornamelijk in het kader van biomedische vraagstellingen. Vooral de fysiologische en anatomische gelijkenis van het varken met de mens maar ook de vergelijkbare hersenontwikkeling rondom de geboorte maken de soort geschikt voor cognitief translationeel onderzoek.

De big als model voor baby's met een laag geboortegewicht

Een van de uitgangspunten van dit proefschrift was om de cognitieve prestaties van biggen geboren met een LGG te bestuderen. Op deze manier trachtten we meer te weten te komen over de cognitieve gevolgen van LGG bij de mens. Meer specifiek was één van de doelen het bestuderen van het leervermogen en geheugen van biggen met een LGG veroorzaakt door intra-uteriene groei vertraging (Engels: Intra Uterine Growth Restriction; IUGR). Een tweede doel was het bestuderen van de effecten van een mogelijke neuroprotectieve therapie om de verwachtte verminderde cognitieve prestaties van LGG biggen, veroorzaakt door IUGR-gerelateerde hersenschade, te beperken. Er diende aan enkele voorwaarden voldaan te worden om deze doelen te kunnen bereiken.

Allereerst was het vinden van een passende cognitieve gedragstest voor het varken noodzakelijk **(hoofdstuk 2, 3 en 4)**. De meest geschikte test diende verder ontwikkeld en gevalideerd te worden **(appendix I, hoofdstuk 5)**. Vervolgens was het mogelijk met deze cognitieve test de effecten op leervermogen en geheugen van

biggen met een LGG te onderzoeken. Om een verondersteld geschikt therapeuticum, namelijk allopurinol, te kunnen testen in de juiste dosering in biggen met een LGG moesten de farmacokinetische eigenschappen van dit therapeuticum in zeugen en in hun foetussen aan het einde van de dracht worden bepaald. Daarna werden zowel de therapeutische effecten, als ook enkele veiligheidsaspecten van allopurinol in biggen met een LGG onderzocht.

Het spatieel cognitief varkens holeboard

Het ontwikkelen en valideren van een cognitieve test waarmee bepaalde aspecten van het leervermogen en geheugen van varkens onderzocht kon worden bleek noodzakelijk. Het bestaan van dergelijke tests die ook herhaaldelijk betrouwbare en gevalideerde resultaten opleverden bleek namelijk beperkt te zijn **(hoofdstuk 2)**. Voor het ontwikkelen van een cognitieve test voor het varken diende aan diverse basiseisen te worden voldaan. Na bestudering van alle bestaande tests bleek dat het vooral van belang was dat hetgeen de test meet een reflectie is van natuurlijk voorkomende gedragingen van het varken. Uitgaande van deze eis leken enkele typen cognitieve tests bijzonder geschikt te zijn. Voor twee verschillende tests werden testopstellingen ontworpen en onderzocht; een visuele discriminatie **(hoofdstuk 3)** test en een test welke het ruimtelijk inzicht meet **(o.a. hoofdstuk 4)**.

De visuele discriminatietaak bleek minder geschikt te zijn voor het vergelijken van LGG biggen met hun toomgenoten met een gemiddeld geboortegewicht. De test leek minder sensitief te zijn voor subtiele verschillen en niet alle varkens toonden zich gemotiveerd. Doordat de dieren hun keuze volledig op visuele informatie dienden te baseren en de visuele capaciteiten van het varken nog niet volledig in kaart zijn gebracht was het ook twijfelachtig of foute keuzes gebaseerd waren op de cognitieve of visuele capaciteiten van de dieren.

Uiteindelijk bleek van beide onderzochte testen de eerder genoemde test die het ruimtelijk geheugen meet het meest veelbelovend. Deze test was van het type ‘vrije-keuze doolhof’; een “cognitief varkens holeboard”. In een dergelijke test dient een dier een ruimtelijke taak op te lossen, bijvoorbeeld het vinden van één of meerdere voedselbeloningen op verschillende locaties. Diverse vrije-keuze tests voor varkens hebben inmiddels hun functionaliteit bewezen, echter tot op heden werden de resultaten van de uitgevoerde experimenten niet gerepliceerd of verder gevalideerd. **(hoofdstuk 2)** Deze cognitieve test voldeed aan bovenstaande eisen en was geschikt voor automatisering. Deze test werd oorspronkelijk ontwikkeld voor knaagdieren maar later ook voor diverse andere diersoorten. Er is zelfs een versie die geschikt is voor het testen van mensen. We maakten de holeboard taak geschikt voor het testen van varkens door deze aan te passen aan hun natuurlijke gedrag en hun motorische en sensorische capaciteiten. Tevens werd de testopstelling uiteindelijk zo aangepast dat alle gedragsmetingen geautomatiseerd geregistreerd konden worden wat de betrouwbaarheid van de resultaten ten goede komt **(hoofdstuk 5)**.

Het cognitieve varkens holeboard (gevisualiseerd in **appendix II**) is een testopstelling waarbij het varken via één van de vier toegangsdeuren een

symmetrische ruimte betreedt en zich hier binnen kan oriënteren aan de hand van aanwijzingen die hij ziet, hoort of ruikt buiten de opstelling. In deze ruimte bevinden zich 16 voerbakken welke afgedekt zijn met een bal welke door een frame op hun plaats wordt gehouden. Door met de snuit de bal op te tillen kan het varken zien of er een beloning in de voerbak is geplaatst en deze vervolgens opeten. Door ieder individueel dier een eigen vaste 'configuratie' aan te bieden van beloningen (vier beloonde versus 12 onbeloonde voerbakken) en hem de gelegenheid te geven de ruimte regelmatig te betreden kan hij leren waar zich wel en geen beloningen bevinden. Daardoor neemt het aantal fouten dat een dier maakt (bezoeken aan bakken zonder beloning of aan bakken waar de beloning al gevonden was) over de tijd af en kan onder andere een leercurve worden bepaald **(hoofdstuk 4, 5 en 6)**.

In **hoofdstuk 4** werden de eerste stappen gezet om LGG biggen te onderzoeken in het cognitieve varkens holeboard. Biggen met LGG zijn vergeleken met biggen met een gemiddeld geboortegewicht, uit dezelfde toom en met hetzelfde geslacht. Beide groepen bleken prima in staat om de aangeboden configuraties te leren en maakten snel minder fouten. De LGG biggen lieten echter na het aanbieden van een tweede –nieuwe– configuratie (reversal; een andere combinatie van beloonde voerbakken) een vertraagde acquisitie van de nieuwe opdracht zien, maar deze achterstand bleek van voorbijgaande aard. Deze resultaten staven de hypothese dat een LGG gerelateerd is aan milde cognitieve beperkingen.

Om het cognitieve varkens holeboard verder te valideren en om inzicht te krijgen in de verschillen in leervermogen en geheugen tussen conventionele vleesvarkens en Göttingen minivarkens (welke vaak worden gebruikt binnen het biomedisch onderzoek) werd een validatiestudie met de stof scopolamine uitgevoerd **(appendix I)**. Scopolamine (een niet-selectieve muscarine receptor antagonist) wordt gezien als de gouden standaard voor het farmacologisch induceren van geheugen beperkingen in gezonde mensen en dieren. Echter, subcutane toediening van scopolamine in het varken bracht diverse moeilijkheden met zich mee en de resultaten bleken moeilijk te interpreteren. Daarom werd een vervolgstudie uitgevoerd met de stof biperiden, welke oraal toegediend kon worden **(hoofdstuk 5)**.

Biperiden (een muscarine M1 receptor blokker) wordt gebruikt om tijdelijke cognitieve beperkingen te induceren in dierexperimenteel onderzoek. Beiden groepen varkens werden in het cognitieve holeboard getest na behandeling met verschillende doseringen biperiden. Voorafgaand aan de behandelingen werden zij eerst getraind tot een stabiel prestatie niveau. Beide groepen varkens bleken in staat om de taak te leren (de minivarkens leerden de taak alleen in een langzamer tempo) en behaalden hetzelfde hoge asymptotische prestatieniveau na ca. 100 trials (pogingen). Behandeling met biperiden leidde onder andere tot een langere trial duur. Het lange termijn geheugen, maar niet het korte termijn geheugen van de dieren verslechterde vanaf een dosering van tenminste 5 $mg.kg^{-1}$. Om de effectiviteit en juiste dosering van biperiden in varkens te kunnen bevestigen is verder onderzoek echter noodzakelijk.

Prenatale neuroprotectie bij laag geboortegewicht biggen

Een tweede uitgangspunt van dit proefschrift was het bestuderen van de effecten van een mogelijke prenatale neuroprotectieve therapie die de cognitieve schade gerelateerd aan LGG in biggen en uiteindelijk baby's zou kunnen beperken of voorkomen. Een LGG op zich is geen aandoening maar het gevolg van ondergemiddelde prenatale groei. Deze groeibeperking kan diverse oorzaken hebben waaronder bijvoorbeeld maternaal overgewicht of alcoholgebruik maar de meest voorkomende oorzaak is placentaire insufficiëntie. Algemeen kan worden gesteld dat een foetus zich aanpast aan een verminderde placentaire werking (minder toevoer van zuurstof en voedingsstoffen) en langzamer groeit, met als eerste zichtbare gevolg een laag geboortegewicht. Er kan echter bijkomende schade in onder andere de hersenen zijn opgetreden, al zijn de mechanismen achter het ontstaan van deze schade nog relatief onbekend. Wanneer het brein met een gebrek aan zuurstof te maken krijgt kunnen hersencellen beschadigen of zelfs sterven. Dit fenomeen is veelvuldig bestudeerd in baby's, biggen maar ook andere diersoorten waarbij perinatale asfyxie voorkomt. Dit fenomeen kan in proefdieren ook experimenteel worden geïnduceerd. In groeivertraagde dieren is de verwachting dat het zuurstofgebrek over het algemeen milder is dan bij acute perinatale asphyxie. De periodes waarin zich een verlaagde zuurstoftoevoer voordoet kunnen echter langduriger zijn en kunnen worden afgewisseld met periodes van reoxygenatie. Wanneer zuurstof de hersencellen weer kan bereiken kan oxidatieve stress optreden en de daarbij geproduceerde vrije radicalen kunnen op hun beurt schade aan de cellen veroorzaken. Dit is waar interventie mogelijk van nut kan zijn; farmacologische behandeling zou schade kunnen voorkomen of beperken en zo de cognitieve vermogens van deze biggen of kinderen verbeteren.

Allopurinol

In de **hoofdstukken 6 en 7** werd een studie uitgevoerd waarbij gekeken is naar de effecten van een mogelijk neuroprotectieve stof, namelijk allopurinol. Van allopurinol is bekend dat het de formatie van schadelijke vrije radicalen kan beperken in diverse diersoorten en de mens. Tevens werd de stof al ingezet in een klinische trial rondom perinatale asphyxie. Allopurinol is in staat de placenta te passeren. Dit is een voorwaarde voor behandeling van een foetus via de moeder of zeug. Tevens is de metaboliet van allopurinol, genaamd oxypurinol, eveneens werkzaam. De veronderstelde therapeutische spiegels voor allopurinol en oxypurinol zijn respectievelijk >2 $mg.L^{-1}$ en >4 $mg.L^{-1}$. Voor het bepalen van de juiste orale dosering bij langdurige toediening gedurende het laatste trimester van de dracht werd voorafgaand aan het hoofdexperiment een klein farmacokinetisch experiment uitgevoerd. Met behulp van deze data werd de dagelijkse dosering voor de zeug vastgesteld op 15 $mg.kg^{-1}$ om hiermee therapeutische plasmaconcentraties in de foetussen te kunnen bereiken.

In twee batches werden zeugen behandeld met allopurinol gedurende het laatste trimester van de dracht. Er werden evenveel controle zeugen ingezet welke onbehandeld bleven. De biggen van de betreffende zeugen zijn direct na de partus gemonitord (o.a. gewicht, geslacht, bloedgassen uit de navelstreng, placenta). Uit deze groep biggen zijn vervolgens zowel dieren met een laag- als met een gemiddeld geboortegewicht geselecteerd welke na het spenen twee gedragstesten

ondergingen. Eenmalig werden de biggen getest in een ‘open ruimte & onbekend object’ test om een indicatie te krijgen van hun emotionele reactiviteit. Vervolgens werden dezelfde dieren gedurende een langere periode getraind in het cognitieve varkens holeboard. Uiteindelijk zijn na het gedragsonderzoek ook de hersenen van deze dieren na 5-5.5 maand uitgeprepareerd en is er gekeken naar de expressie van voor cognitie relevante markers. Deze markers (o.a. mBDNF en PS95) bevinden zich in de Brain Derived Neurotrophic Factor (BDNF) neuronale plasticiteit route in het dorsale deel van de hippocampus.

Behandeling met allopurinol heeft noch nadelige effecten laten zien tijdens het laatste trimester van de dracht, noch op het geboorteproces. De placenta’s van behandelde biggen vertoonden geen relevante verschillen ten opzichte van placenta’s van controle dieren. Wel werden er correlaties gevonden tussen een laag geboortegewicht enerzijds en een lager placentagewicht en een kleinere placenta omtrek anderzijds.

We vonden nauwelijks indicaties voor verschillen in emotionele reactiviteit tussen behandelde en onbehandelde dieren. Biggen met een laag geboortegewicht vocaliseerden echter meer dan hun toomgenoten met een gemiddeld geboortegewicht. Dit duidde mogelijk op een verhoogd angstniveau in de biggen met een laag geboortegewicht. Aangezien vergelijkbare observaties gedaan zijn in kinderen is dit waarschijnlijk een gedeeld fenomeen tussen beide soorten.

In het cognitieve varkens holeboard werden geen verschillen gevonden tussen met allopurinol behandelde dieren en controledieren, maar ook niet tussen dieren met een laag en een gemiddeld geboortegewicht. De bevindingen uit **hoofdstuk 4**, waar we concludeerden dat biggen met een laag geboortegewicht minder flexibiliteit toonden rondom de overgang van één geleerde configuratie naar een nieuwe, werden in dit experiment niet bevestigd. Diverse verschillen in de opzet van het experiment kunnen hiervan de oorzaak zijn. Desondanks geeft deze studie een goede indicatie dat langdurige orale toediening van allopurinol via de zeug aan biggen met een laag en gemiddeld geboortegewicht veilig kan worden geacht voor de cognitieve ontwikkeling en ook verder geen ongewenste bijeffecten heeft.

Het uiteindelijke lichaams- brein en hippocampusgewicht van de varkens met een laag geboortegewicht bleef na 5-5.5 maand gemiddeld lager dan dat van toomgenoten met een gemiddeld geboortegewicht. Gekeken naar het relatieve brein en hippocampusgewicht (lichaamsgewicht/brein of hippocampus gewicht) van de dieren werd duidelijk dat er wel sprake was van enige postnatale compensatie van groei. Relatief waren de hersen- en hippocampusgewichten van de LGG dieren hoger dan die van de dieren met een normaal geboortegewicht. Van de dieren behandeld met allopurinol lieten de LGG dieren een compensatie van groei zien (lichaamsgewicht). Ook in dit opzicht kan geconcludeerd worden dat het gebruik van allopurinol in deze toepassing veilig lijkt en dat het wellicht de aan LGG gerelateerde cognitieve deficiënties kan beperken. De effectiviteit van allopurinol in deze toepassing behoeft echter verder onderzoek.

Conclusies en aanbevelingen

- Na automatisering en een validatie studie is het varkens holeboard een betrouwbare taak gebleken voor het meten van diverse componenten van leervermogen en geheugen van het varken. Het cognitieve holeboard behoort tot de eerste valide cognitieve tests voor het varken.
- Biggen geboren met een LGG blijken een veelbelovend diermodel te zijn voor het nabootsen van IUGR in humane baby's. Het model dient echter nog verder verfijnd en gevalideerd te worden.
- Studies met het cognitieve varkens holeboard hebben laten zien dat deze taak geschikt is om de spatiele capaciteiten rondom leervermogen en geheugen van biggen met LGG te kunnen meten. Biggen met LGG bleken minder flexibel te zijn dan controle dieren met een gemiddeld geboortegewicht wanneer ze een nieuwe configuratie van beloonde voerbakken gepresenteerd kregen. Deze inflexibiliteit was van tijdelijke aard. In een volgende studie met LGG dieren werden deze resultaten echter niet gerepliceerd, mogelijk als gevolg van diverse verschillen in de opzet van de tweede studie.
- Een veiligheids- en effectiviteitsstudie van de mogelijke neuroprotectieve stof allopurinol wees erop dat chronisch gebruik bij het varken veilig is tijdens het laatste trimester van de dracht, geen negatieve invloed heeft op de partus en de cognitieve vermogens van de varkens niet negatief beïnvloedt.
- Prenatale chronische behandeling met allopurinol gedurende het laatste trimester van de dracht leverde geen verschillen in prestatieniveau in het cognitieve varkens holeboard tussen behandelde en controledieren met een laag en normaal geboortegewicht op. Echter, in de dorsale hippocampus van beide groepen behandelde dieren werd een verhoogd proteïne niveau van zowel mBDNF als PS95 gevonden. Deze resultaten wijzen op een positieve invloed van allopurinol op de ontwikkeling van de hippocampus, welke een belangrijke rol speelt bij cognitie.
- Verder onderzoek met zowel allopurinol als LGG biggen strekt tot aanbevelingen. Daarnaast zou verfijning van het LGG biggen model, bijvoorbeeld door het intensiveren van de neonatale zorg voor biggen de validiteit van het model ten goede komen. Het cognitieve varkens holeboard is een geschikte test gebleken om in te zetten binnen dit LGG model en de opgedane kennis zou tevens kunnen bijdragen aan het welzijn van biggen geboren met LGG.

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About the author

Elise Gieling was born on March 14, 1983 in Zeist (The Netherlands). After attending Norbertus College high school in Roosendaal from 1995 to 2001, she studied Animal Husbandry at the University of Applied Sciences, HAS Den Bosch ('s-Hertogenbosch) from 2001 to 2005 and specialised in animal health. After this Bachelor an Animal Sciences Master at Wageningen University and Research (Wageningen) followed from 2005 to 2007. During her studies, Elise was busy organizing a yearly equestrian event focussing on horse behaviour and welfare and for her Bachelor internship she studied Przewalski horses in national park Hustai Nuuru, Mongolia, for three months. The major and minor research projects of her Master studies were both conducted at the Faculty of Veterinary Medicine (Utrecht) at the department of Animals in Science and Society.

After her graduation Elise worked at the National Center for Alternatives to Animal use (currently NCKA) and as a teacher at a school for intermediate vocational education (Edudelta College, Barendrecht). During this year she additionally followed a Postgraduate education in Applied Animal Behaviour at the University of Applied Sciences, KaHo St. Lieven (St. Niklaas, Belgium). In August 2008, one year after graduation, she started her PhD studies with pigs as model for low-birth-weight babies at the department of Farm Animal Health, Emotion and Cognition group, Faculty of Veterinary Medicine, Utrecht University.

List of publications

Refereed scientific publications

Arndt SS, Laarakker MC, van Lith HA, van der Staay FJ, Gieling E, Salomons AR, van 't Klooster J, Ohl F (2009) Individual housing of mice – impact on behaviour and stress responses. Physiol Behav 97:385-393

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Contributions to conferences and symposia

Gieling ET, Cox ME, van Dierendonck MC (2007) Group housing with automatic feeding systems: implications for behavior and horse welfare. Proceedings of the 3rd International Equitation Science Conference, Michigan, USA

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Gieling ET, Nordquist RE, van der Staay FJ (2011) Growth restricted piglets: a natural occurring animal model of Intra Uterine Growth Restriction in humans? Accepted abstract NIDA Overseas Investigator Travel Award, Hollywood (FL), USA

Gieling ET (2011) Growth restricted piglets: a naturally occurring potential animal model of Intra Uterine Growth Restriction in humans. Accepted abstract summerschool „Does Size Matter?“ (Europäische Akademie zur Erforschung von Folgen wissenschaftlich-technischer Entwicklungen), Munich, Germany

Gieling ET, Wehkamp W, Willigenburg R, Nordquist RE, Ganderup N-C, van der Staay FJ (2011) Training and anticholinergic treatment (biperiden) of pigs in the cognitive holeboard: a comparison study between Göttingen mini- and conventional pigs. Abstract Minipig Research Forum 2011, Frankfurt, Germany

Miscellaneous

Gieling ET, Nordquist RE, Arts J, van der Staay FJ (2012) Meten van ruimtelijk leren en geheugen van varkens in de holeboardtaak. Biotechniek 51:19-23

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