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The postnatal development of neocortical neurons and glial cells in the Göttingen minipig and the domestic pig brain

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Summary

The first mathematically unbiased estimates of neocortical cell numbers are presented from the developing pig brain, including a full description of tissue processing and optimal sampling for application of the stereological optical fractionator method in this species. The postnatal development of neocortical neurons and glial cells from the experimental Göttingen minipig was compared with the postnatal development of neocortical neurons in the domestic pig. A significant postnatal development was observed in the Göttingen minipig brain for both neuronal (28%; *P*=0.01) and glial cells (87%; *P*<0.01). A corresponding postnatal development of neurons was not detected in the domestic pig brain. The reason for this strain difference is not known. The mean total number of neocortical neurons is 324 million in the adult Göttingen minipig compared with 432 million in the domestic pig. The glial-to-neuron cell ratio is around 2.2 in the adult Göttingen minipig. Based on these results, the domestic pig seems to be a more suitable model for evaluating the effects of developmental insults on human brain growth and neuronal development than the Göttingen minipig.

Key words: cavalieri volume, fractionator, porcine, total cell number, stereology.

Introduction

The Göttingen minipig and the domestic pig are increasingly used as non-primate models in basic experimental studies of neurological diseases. The gyrated pig brain is more similar to the primate brain than lissencephalic brains from small laboratory animals. The pig is affordable, it is easily handled and its use may potentially avoid some of the ethical considerations concerning the use of primates as laboratory animals.

The pig has previously been considered a potential animal model for evaluating the effects of developmental insults on human brain growth and development (Dickerson and Dobbing, 1966; Book and Bustad, 1974; Dobbing and Sands, 1979; Pond et al., 2000). In general, the mammalian brain appears to go through a momentary period of rapid growth, exemplified by a characteristic growth rate curve when the percent of adult brain mass is plotted against age (Dobbing and Sands, 1979). One of the most important interspecies differences seems to be the complexity of the final product as well as the timing of the brain growth spurt in relation to birth (Dobbing, 1974). The timing of the brain growth spurt can be used to roughly categorize mammalian species as prenatal, perinatal or postnatal developers (Dobbing and Sands, 1973). In a comparison of seven mammalian species, it was demonstrated that the pig brain, like that of humans, develops perinatally, with a brain growth spurt extending from midgestation to early postnatal life (Dickerson and Dobbing, 1966; Dobbing and Sands, 1979; Pond et al., 2000). This is in contrast to other mammalian species, e.g. the brain of guinea pig, sheep and monkey, which has a prenatal growth spurt, or the brain of rat and rabbit, which develops postnatally (Dobbing and Sands, 1979). The development of the pig brain is also considered more similar to the human brain with respect to myelination, compositions and electrical activity (Dickerson and Dobbing, 1966; Fang et al., 2005; Flynn, 1984; Pampliglione, 1971; Thibault and Margulies, 1998).

The traditional view of the primate neocortex is that it is structurally stable and that neurogenesis and synapse formation occur during early development (Bourgeois et al., 1994; Rakic, 1985b). Quantitative studies based on DNA quantification in the human brain have indicated that the major phase of neuronal multiplication occurs during the first half of gestation, prior to the numerically larger but slower phase of glial multiplication, which continues into the first postnatal years (Dobbing and Sands, 1973; Dobbing, 1974). A two-phased growth pattern has similarly been observed in a stereological study on total cell

numbers in the developing human fetal forebrain (Samuelsen et al., 2003). A clear cell discrimination has, however, not been performed in early fetal life.

In an attempt to further evaluate the pig as a potential model for human brain development and to provide a quantitative structural basis for comparative and experimental studies, a number of quantitative examinations on the neonate and adult pig brain have been initiated. Quantitative data are obtained using assumption-free stereological methods. The methods are designed to describe quantitative parameters without assumptions about shape, size, orientation and distribution of cells in the reference space and are based on established procedures for systematic, uniformly random sampling that are superior in precision compared with results obtained by independent sampling (Gundersen and Jensen, 1987). In the present paper, neocortical cell numbers were obtained using the optical fractionator method (Gundersen, 1986; West et al., 1991). A detailed description of tissue processing and optimal sampling procedures for application in the pig brain is presented.

Materials and methods

Experimental animals

The Göttingen minipig

The Göttingen minipig was developed in 1961–1962 at the Institute of Animal Breeding and Genetics of the University of Göttingen (Germany). The present characteristics of the Göttingen minipig, as a small, white miniature pig with good fertility and stable genetics, were obtained as a result of cross-breeding the Minnesota minipig with the Vietnamese potbelly pig and the German Landrace (Bollen and Ellegaard, 1997; Damm Jorgensen, 1998). The Göttingen minipig in Denmark originates from caesarean sections performed on German sows in the early 1990s. The offspring was transferred to a barrier facility and kept in a non-contaminated environment. Only three populations of limited population size exist in the world today.

The Göttingen minipig is an outbred animal, with less than 5% in-breeding (Glodek, 1986). The newborn Göttingen minipig has a body mass of ~350–450 g. Boars become sexually mature at an age of 3–4 months, weighing 6–8 kg, while sows are sexually mature at an age of 4–5 months, weighing 7–9 kg. The gestational period is 112–114 days and the average litter size is 5–6 animals. The adult mass of the Göttingen minipig, at an age of two years, is 35–40 kg (Bollen and Ellegaard, 1997; Damm Jorgensen, 1998).

The Göttingen minipig of today is not gnotobiotic but is kept in barriers to avoid bacterial contamination and thereby minimize the risk of any influence on research caused by microbiological organisms. Health monitoring is carried out twice a year, based on FELASA (Federation of European Laboratory Animal Science Associations) guidelines.

The domestic pig

The domestic pigs used in the present study are all crossbreeds between Danish Landrace and Yorkshire. This combination is normally used for production of mother animals. Danish Landrace is a long and lean breed known for high fertility and good motherhood. The litter size is around 13.5 piglets per litter. The Yorkshire pig has a high lean meat percentage, high daily gain and good meat quality. The mothering characteristics are good, with a litter size of around 12 piglets per litter. The National Committee for pig production mainly organizes The Danish Pig breeding program.

The newborn Landrace/Yorkshire domestic pig has a body mass of ~1.3–1.9 kg. Boars become sexually mature at an age of 6 months, while sows are sexually mature at an age of 7 months. The gestational period is 114 days and the average litter size is 10–14 animals. The adult mass of the domestic pig, at an age of two years, is 200–300 kg (Bollen et al., 2000). In Denmark, most of the domestic pigs are SPF-tested (Specific Pathogen Free) but are not submitted to the same strict control procedures as the Göttingen minipigs.

Experimental set-up

A total of 10 Göttingen minipigs and 12 domestic pigs were used in the study. All Göttingen minipigs [five neonate polts (1–2 days old) and five adult sows (1.5–3 years old)] were perfusion fixated by a procedure approved by the Danish Animal Research Inspectorate. The pigs were anesthetized with an intramuscular injection (1 ml per 10 kg body mass) of a mixture of 6.5 ml Narcoxyl®Vet (20 mg ml^{-1}; Intervet, Denmark), 1.5 ml Ketaminol®Vet (100 mg ml^{-1}; Intervet, Denmark) and 2.5 ml Methadone DAK (19 mg ml^{-1}; Nycomed, Denmark) added to one bottle of Zoletil®50 Vet (Virbac Laboratories, France) without additional solvent. The pigs were then placed in a supine position on the operation table and supplied with a lethal dose of Pentobarbital (1 ml kg^{-1} body mass, 200 mg ml^{-1}) before intervention. The deeply 'anesthetized' pigs received a midsternal incision followed by a sternal split. The left cardiac ventricle was punctured by an infusion cannula, the right auricle was cut open, and a transcardial perfusion with 0.5–2.5 l saline followed by 2.5–7.5 l of 4% paraformaldehyde in 0.15 mol l^{-1} Sørensen phosphate buffer (pH 7.4) was executed. The procedure took on average 10–15 min. The brain was removed and postfixed for an additional 24 h at 5°C in 1% paraformaldehyde.

The domestic pigs [six neonate sows (1 day old) and six adult sows (3–4 years old)] were euthanized with a lethal injection of Pentobarbital i.v. or with carbon dioxide in a Danish pig slaughterhouse. Following death, the brains were carefully removed from the skull and fixed by immediate immersion in 4% formaldehyde (0.15 mol l^{-1} phosphate buffer, pH 7.4) for at least two weeks.

Tissue processing

The cerebral hemispheres were divided medially through the corpus callosum, and the left or right hemisphere was selected randomly before dehydration and infiltration in paraffin (Fig. 1A,B). The hemispheres were placed in a container with the midsagittal surface facing down, embedded in blocks of paraffin and sectioned into coronal series of 40 μm-thick sections on a Leica microtome (Fig. 1B,C). Satisfactory

adhesion of the thick sections to glass slides was obtained using Superfrost Plus glass slides primed with a chromalun–gelatin solution (Feinstein et al., 1996). A predetermined fraction of the 40 μm-thick sections was sampled using moist filter paper and carefully rolled onto 40°C preheated primed slides covered by a thin layer of distilled water. The sections were dried overnight at 37°C and a final selection of slides was subsequently stained with Giemsa.

Stereological techniques

Estimates of neocortical cell numbers were obtained using the optical fractionator method (Fig. 1). The optical fractionator combines two stereological principles: the optical disector for counting particles and the systematic uniform sampling scheme of the fractionator (Gundersen, 1986; West et al., 1991). The optical fractionator involves counting particles with optical disectors in a uniform random systematic sample that constitutes a known volume fraction of the region being analyzed. This is achieved by counting cells on a known fraction of sections (*ssf*; Fig. 1D), under a known fraction of the sectional area of the region (*asf*; Fig. 1E) in a known fraction of the thickness of a section (*hsf*; Fig. 1G). The total numbers of neocortical cells are estimated by multiplying the numbers of cells counted (ΣQ^-) with the inverse sampling fractions. Finally, the bilateral cell number can be estimated by multiplying the unilateral number by two.

$$N_{\mathrm{total}} = (1/ssf)\,(1/asf)\,(1/hsf)\,\Sigma Q^{-}\,2\,. \quad (1)$$

The optical disector may be considered as a three-dimensional probe, generated with the aid of a microscope with a high numerical aperture (NA=1.4) and an oil immersion objective, in which it is possible to observe thin focal planes in relatively thick sections (Fig. 1G). A counting frame with 'exclusion' and 'inclusion' lines is superimposed on the magnified image of the tissue on a computer screen, and the orientation in the *z*-axis is measured with a digital microcator with a precision of 0.5 μm. The purpose of 'exclusion' and 'inclusion' lines of the counting frame is to exclude edge effects arising from sub-sampling (Gundersen, 1978). Similarly, upper and lower guard zones protect the counting frame to prevent

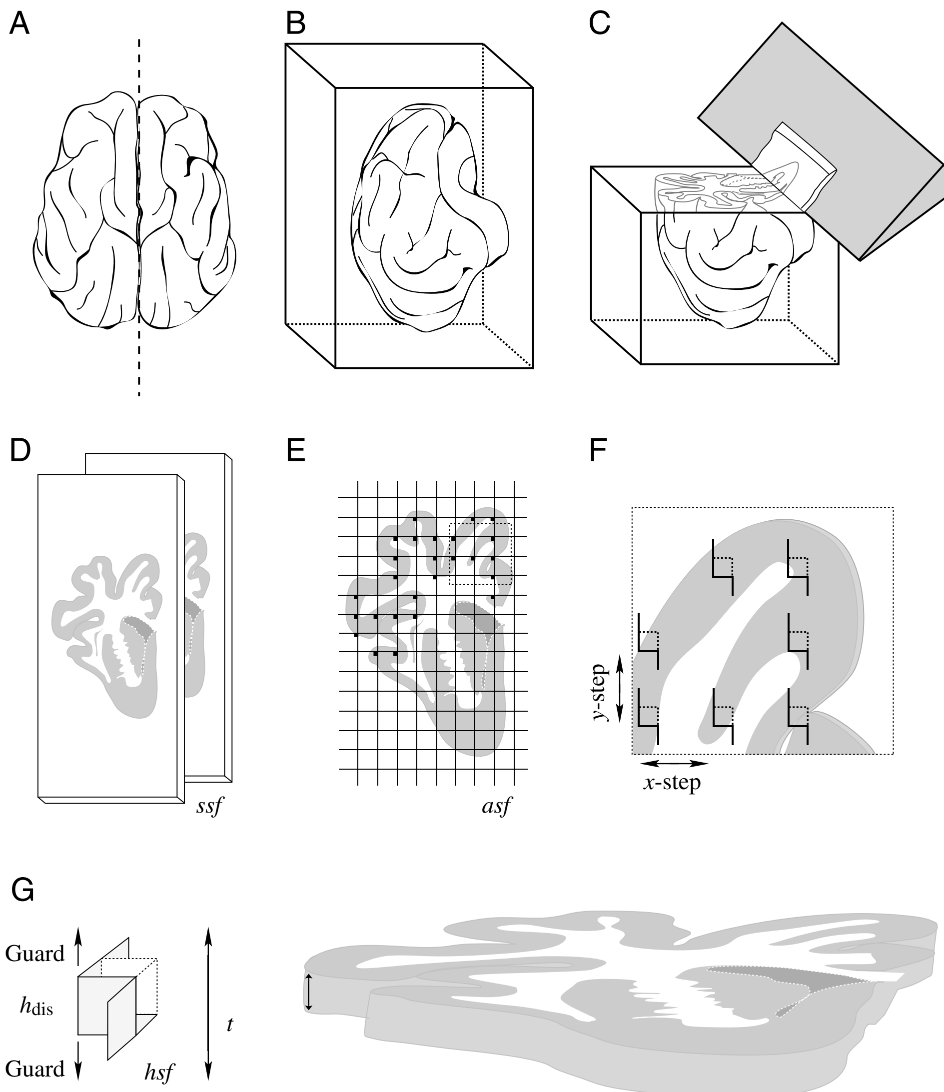

Fig. 1. The optical fractionator sampling scheme. The cerebral hemispheres were divided medially (A), and the left or right hemisphere was selected systematically randomly before dehydration and infiltration in paraffin (B). The hemispheres were sectioned exhaustively into 40 μm-thick coronal sections (C), from which a predetermined fraction, the section sampling fraction (*ssf*), was sampled systematically randomly (D) and subsequently stained with Giemsa. Optical disectors were positioned systematically randomly on each of the sampled sections at regular predetermined *x*,*y*-positions (E,F). The area of the counting frame of the disector to the area associated with each *x*,*y*-step represents the area sampling fraction (*asf*). Counts were performed in all optical disectors hitting the structure of interest (E,F). Cellular nuclei were counted by moving the counting frame through a continuous stack of thin optical planes inside the section (G). The height of the disector (h_{dis}) relative to the height of the section (*t*) represents the height sampling fraction (*hsf*). The disector is protected by upper and lower guard zones.

Table 1. *The fractionator sampling parameters, mean values*

	BA (μm)	1/*ssf*	Σsect	*a(frame)* (μm²)	Step size (*x*,*y*) (μm)	1/*asf*	Σdis	h_{dis} (μm)	t_{Q^-} (μm)	1/*hsf*	ΣQ^-_{neu}	ΣQ^-_{glial}
Göttingen minipig												
Neonate	40	50	11–13	749	1600	3417	172	15	39.1	2.6	253	383
Adult	40	100	11–12	2497	2600	2707	168	20	37.7	1.9	289	337
Domestic pig												
Neonate	40	100	8–9	999	1800	3242	141	15	38.4	2.6	260	–
Adult	40	200	7–8	2706	2800	2896	137	25	37.9	1.5	235	–

Abbreviations: BA, block advance; *ssf*, section sampling fraction; Σsect, number of sections; *a(frame)*, area of counting frame; Σdis, number of disectors hitting neocortex; h_{dis}, disector height; t_{Q^-}, q-weighted section thickness; *hsf*, height sampling fraction; ΣQ^-_{neu}, number of neurons counted; ΣQ^-_{glial}, number of glial cells counted.

The *a(frame)* for glial cell estimations in adult Göttingen minipigs was 50% of the original frame.

bias as a consequence of loss of cells close to the section surfaces (Fig. 1G). All cells that come into focus within the frame and are not in focus at the uppermost position are counted as the focal plane is moved through the section. All cell counts were obtained using a BH-2 Olympus microscope and CAST-GRID software (Olympus, Ballerup, Denmark).

Counting criteria

The cells were identified as neurons if they had a combination of dendritic processes, a Giemsa-positive cytoplasm, a clearly defined nucleus with a pale and homogeneous nucleoplasm and a dark and condense centrally located nucleolus. The nucleus was used as the counting item, and around 250 neuronal nuclei were counted per brain (ΣQ^-_{neu}; Table 1). The glial cells were usually smaller and identified by the absence of a Giemsa-positive cytoplasm, the presence of heterochromatin clumps within the ovoid or irregularly shaped nucleus and the lack of a clearly identifiable nucleolus. Also here, the nucleus was used as the counting item, and an average of 360 glial nuclei were counted per brain (ΣQ^-_{glial}; Table 1). No differentiation was made between astrocytes, oligodendrocytes or microglia. Endothelial cells were easily recognized by their dark and elongated nucleus and were excluded from all counts.

The section sampling fraction

A sampling scheme was designed based on systematic uniform random sampling (SURS) to ensure that all parts of the neocortex had an equal probability of being sampled. Based on a pilot study, every 50th or 100th section was sampled from the neonate and adult Göttingen minipig brain, respectively, whereas every 100th or 200th section was sampled from the domestic pig (Table 1). The first section was selected randomly using a random number within the sampled section period. In the event of poor technical quality, e.g. due to the presence of folds or breaks, the subsequent section was sampled instead. Extra sections were also sampled to circumvent potential damage of sections during staining procedures. These deviations are of no consequence to the results, provided the overall sampling scheme is maintained. The section fraction represents the section sampling fraction (*ssf*) and provided 11–13 sections for final quantitative analyses in the Göttingen minipig and 7–9 sections in the domestic pig.

The area sampling fraction

In each of the sampled sections, counts of neurons or glial cells were made with optical disectors at regular predetermined *x*,*y* positions in the neocortex. The neocortex was defined as the isocortex and mesocortex. Again, the first disector was positioned randomly within the first *x*,*y* interval by the CAST-GRID software. The area of the counting frame of the disector, *a(frame)*, is known relative to the area associated with each *x*,*y*-step. The area of the sampling fraction (*asf*) is accordingly:

$$asf = a(frame) \,/\, a(x,y\text{-step}) \,. \qquad (2)$$

In a pilot study, the step length between disectors was adjusted to provide approximately 150 disectors in each brain. Subsequently, the frame area and height of the disector were adjusted to obtain 1–2 particles per disector (see below).

The height sampling fraction

The height of the disector (h_{dis}) should be known relative to the thickness (t) of the section. To prevent bias as a consequence of loss of cells, the disector is guarded by upper and lower guard zones. The size of the guard zones depends upon the size of the particles counted. For neocortical neurons it should usually be at least 5 μm in the top and bottom of the section. To compensate for potential deformation of the sections in the *z*-axis, the height sampling fraction (*hsf*) depends on the Q^- weighted mean section thickness ($\bar{t}_{Q^-}$):

$$hsf = h_{dis} \,/\, \bar{t}_{Q^-} \,, \qquad (3)$$

$$\bar{t}_{Q^-} = \Sigma(t_i q_i^-) \,/\, \Sigma(q_i^-) \,, \qquad (4)$$

where t_i is the local section thickness centrally in the *i*th counting frame with a disector count of q_i^- (Dorph-Petersen et al., 2001).

Volume estimates

The systematic uniform random placement of disectors in the fractionator design was used to estimate volumes in

Table 2. *Litter parameters and major estimated quantities*

	N	Body mass (kg)	Brain mass (g)	Neocortex volume (cm^3)	Mean CE volume	Neocortical neurons ($\times 10^6$)	Mean CE neurons	Neocortical glial cells ($\times 10^6$)	Mean CE glial cells
Göttingen minipig									
Neonate	5	0.56 (0.081)	27.8 (0.037)	1.75 (0.057)	0.049	252.5 (0.11)	0.065	381.9 (0.10)	0.053
Adult	5	37.9 (0.11)	79.0 (0.077)	9.01 (0.11)	0.052	323.8 (0.093)	0.064	714.2 (0.12)	0.057
P-value		–	–	–	–	0.01	–	<0.01	–
Domestic pig									
Neonate	6	214.5 (0.30)	29.4 (0.16)	3.64 (0.098)	0.060	424.8 (0.13)	0.067	–	–
Adult	6	212-217	134.0 (0.11)	17.2 (0.083)	0.051	432.1 (0.046)	0.068	–	–
P-value		–	–	–	–	0.76	–	–	–

Mean CE=$\sqrt{\text{mean}(CE^2)}$. For calculation of CE for cell number and volume, see Table 3. The inter-individual variability in each group is shown in parentheses as coefficient of variability (CV=s.d./mean). Volume estimates are from shrunken paraffin-embedded tissue and should not be compared with fresh brain volumes.

accordance with the unbiased principles of the Cavalieri-estimator:

$$V_{neo} = 2\,\Sigma P\,a(p)\,t\,k\,, \quad (5)$$

where ΣP is the number of frame upper-corner-points hitting the neocortex, $a(p)$ is the x,y-step area, t is the block or microtome advance and k is the section sampling fraction. Note that estimated volumes are not used for the estimation of total cell number; the fractionator estimates of total cell number are independent of the containing volume and its shrinkage and deformation.

Statistical analyses and estimation of precision

The differences between means were analyzed using an unpaired two-tailed Student's *t*-test. Group variability is shown in parentheses as the coefficient of variation (CV=s.d./mean) (Table 2). The precision of the estimate of the total cell number in each subject was estimated as the coefficient of error (CE=s.e.m./mean), caused by sampling error related to the counting noise, the systematic uniform random sampling and variances in section thickness (Table 3). The precision of an individual estimate is related to the uniformity of the distribution of particles being counted and the amount of sampling that has been performed (Table 3). The sampling was considered optimal when the observed variance of the individual estimate, CE^2, was less than half the observed interindividual variance, CV^2.

Results

The total number of neocortical neurons in the Göttingen minipig brain increases from ~253 million at birth to ~324 million in adulthood (Table 2, Fig. 2A). This significant (P=0.01) 28% difference demonstrates a pronounced postnatal development of neurons in the Göttingen minipig brain. A significant postnatal development is also observed for neocortical glial cells (P<0.01), increasing from ~382 million in the neonate to ~714 million glial cells in the adult Göttingen minipig, an 87% difference (Table 2; Fig. 2B). The glial-to-neuron ratio changes accordingly from 1.5 to 2.2. The total brain mass increases almost threefold from a mean of 27.8 g at birth to 79.0 g as adult. Meanwhile,

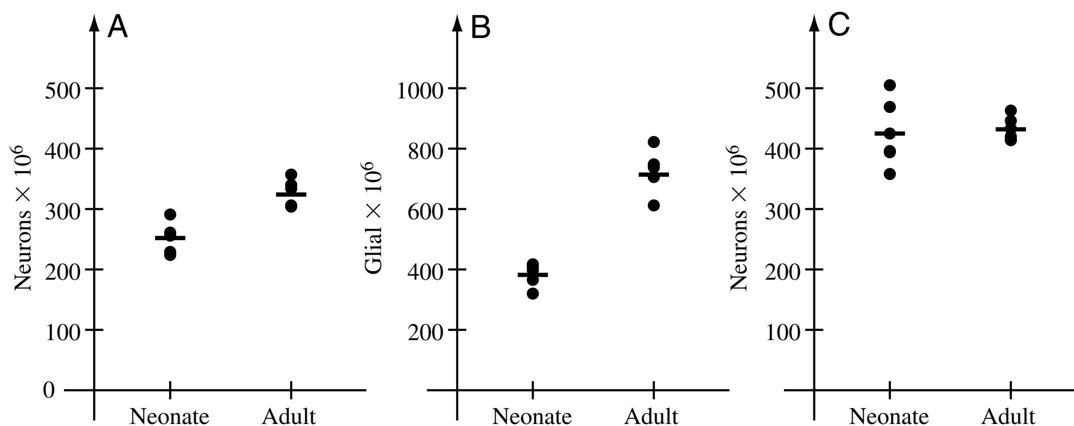

Fig. 2. The postnatal development of neocortical cell numbers in the Göttingen minipig and the domestic pig brain. Total neuron number (A) and total glial cell number (B) in the Göttingen minipig. Total neuron number in the domestic pig (C).

the proportional increase of neocortex volume is more than 500% (Table 2).

A corresponding postnatal development of neocortical neurons is not observed in the domestic pig brain. The domestic pig has ~425 million neocortical neurons at birth and ~432 million in adulthood (Table 2; Fig. 2C), which is ~35% more than in the adult Göttingen minipig neocortex (P<0.01). The number of neocortical glial cells was not estimated. The total brain mass increases from a mean of 29.4 g at birth to 134.0 g as adult. The postnatal proportional increase in neocortex volume is, by comparison, close to 500% (Table 2).

Table 3. *How to estimate the coefficient of error (CE) of the estimate in an individual animal*

Section no.	Q^-	A $Q^-_i \times Q^-_i$	B $Q^-_i \times Q^-_{i+1}$	C $Q^-_i \times Q^-_{i+2}$
1	14	196	224	308
2	16	256	352	288
3	22	484	396	506
4	18	324	414	216
5	23	529	276	552
6	12	144	288	312
7	24	576	624	528
8	26	676	572	884
9	22	484	726	748
10	33	1089	1122	462
11	34	1156	476	
12	14	196		
Σ	258	6110	5470	4778
VAR_{NOISE} [a]	258			
VAR_{SURS} [b]	1.89			
CE(t) [c]	0.007			
CE(ΣQ^-) [d]	0.062			
CE(N) [e]	0.063			

The precision, CE, of a fractionator estimate is a function of three independent factors: the noise variance (VAR_{NOISE}), the systematic uniform random sampling variance (VAR_{SURS}) and the variance attributable to variations in section thickness [CE(t)](see also West and Gundersen, 1990; West et al., 1991; West et al., 1996).

[a]VAR_{NOISE} is the uncertainty' in the estimate that comes from disector counts *within* a section and is equal to ΣQ^-.

[b]VAR_{SURS} is the 'uncertainty' in the estimate that arises due to sampling *between* sections, i.e. because repeated estimates based on different sets of sections may vary. The VAR_{SURS} is calculated using a prediction model that takes into account the systematic nature of the sampling (Gundersen et al., 1999): VAR_{SURS}= [3(A–Noise)–4B+C]/240. For calculation of A, B and C, see columns above. When one uses more than 5–10 sections for a biological estimate, the SURS variance is usually negligible relative to the Noise variance. The denominator is a constant (Gundersen et al., 1999).

[c]$CE(t)=[s.d.(\bar{t})/(\sqrt{n})](1/\bar{x})$, where $\bar{t}$ is the mean section thickness in each section measured with the digital microcator, n is the number of sections and $\bar{x}$ is the mean section thickness between all sections. The CE(t) usually contributes less than 1% to the overall estimator variance and can be ignored in most studies where section homogeneity is high.

[d]The total sampling variance, CE(ΣQ^-), is calculated as: $\sqrt{VAR_{NOISE}+VAR_{SURS}}/\Sigma Q^-$.

[e]The total CE for the final estimate, CE(N), is eventually calculated as: $\sqrt{CE^2(\Sigma Q^-)+CE^2(t)}$.

From the calculations above (a–e), it is obvious that the CE(N) for all practical purposes may be considered a function of the NOISE variance. If one wants to reduce the CEs of the individual estimates further, this would, in this example, be achieved most effectively by sampling more on the sections already in the series.

Discussion

With the principal aim to evaluate the pig as a potential animal model for human brain development, a comparative study has been performed to evaluate the postnatal development of neocortical cell numbers in the experimental Göttingen minipig and the domestic pig brain. It is demonstrated that the cellular development in the Göttingen minipig brain is incomplete at term and that both neocortical neuron and glial cell numbers increase significantly from time of birth to adulthood. By contrast, the neocortical development of neurons in the domestic pig seems to be fully established at birth. An explanation for this strain difference is not at hand.

A rapid development of the brain prior to the general body growth seems to be characteristic for all mammals. Differences between species are related to the gestational time of major cellular multiplication, the developmental stage at birth and the complexity of the final product (Dobbing, 1974). At the time of birth, the human brain constitutes around 23% of its adult mass, and in the first two postnatal years it increases rapidly to around 75% of its adult mass (Dobbing, 1974). In comparison, the brain of the closely related macaque monkey constitutes almost 65% of its adult mass at birth (Dobbing, 1974). The pig brain constitutes around 25% of its adult mass at birth and seems in this aspect to be more similar to that of humans (Dickerson and Dobbing, 1966). The pig is also considered a perinatal brain developer, like human, and several studies have shown a good correspondence to the developing human brain with respect to myelination, compositions and electrical activity (Dickerson and Dobbing, 1966; Pampliglione, 1971; Fang et al., 2005; Flynn, 1984; Thibault and Margulies, 1998). Accordingly, the pig has been considered an appropriate model for human brain development (Dickerson and Dobbing, 1966; Book and Bustad, 1974; Pond et al., 2000).

The present study demonstrates different developmental patterns in the neocortex of two strains of pigs. A significant postnatal development of neuron and glial cells is observed in the Göttingen minipig, whereas the adult number of neurons is established at birth in the domestic pig. This difference in cell number is not represented by a corresponding difference in relative brain mass of the neonates. The brain constitutes 35% of its adult mass in the Göttingen minipig and 22% in the domestic pig, whereas the relative growth of neocortical volume is close to 500% in both strains. The differences can by explained neither by differences in the stereological sampling or counting procedures (see below) nor the different tissue processing. Both fixation methods provided adequate

histological sections with no clear difference in cellular morphology. No clinical data corroborate the observed difference; the gestational period is the same and birth mass and behavior is more or less identical in both strains. However, the results do substantiate that strain differences should be considered in future experimental studies using the pig brain and exemplifies the need for a full designation of the specific strain used.

Based on the results, the domestic pig seems to be a more proper model for evaluating the effects of developmental insults on the human brain than the Göttingen minipig. Even though recent developments have made it possible to unambiguously demonstrate that new neurons are added to the adult primate neocortex as well as to other mammal brains (Gould et al., 1999; Gould et al., 2001; Gould and Gross, 2002), it is still generally accepted that most neurogenesis in monkeys (Rakic and Sidman, 1968; Sidman and Rakic, 1973; Rakic, 1974; Rakic, 1978; Rakic, 1985a; Rakic, 1985b; Rakic, 1988) and humans (Dobbing and Sands, 1973; Dobbing, 1974; Samuelsen et al., 2003) is completed at midgestation or at least before term. The rate of neurons added to the neocortex in adulthood has no relative influence on the total neocortical neuron number when compared with the rate of multiplication during early development (Gould et al., 2001). Furthermore, recent stereological results from our laboratory reveal that the total number of neurons in the cortical plate of human newborns equals the total number in adults (Larsen et al., in press). These results definitively refute previous results demonstrating a major postnatal neurogenesis in humans (Shankle et al., 1998; Shankle et al., 1999). Regrettably, glial cells were not estimated in the domestic pig. Results from one neonate and one adult domestic pig (not presented) do indicate a prenatal development in glial cell number similar to the postnatal increase observed in the Göttingen minipig. Further studies on several postnatal ages are considered valuable in order to describe the growth slope of neuronal increase in the Göttingen minipig.

The Göttingen minipig and the domestic pig have also been considered as useful non-primate models for a number of human neurological diseases (McClellan, 1968; Douglas, 1972). However, although several neuroanatomical studies have been performed, the documentation of pig brain anatomy, connectivity and function is still incomplete. Quantitative information of cell numbers based on systematic sampling procedures has been limited to subcortical areas, e.g. the hippocampus (Holm and West, 1994) and the subthalamic nucleus (Larsen et al., 2004). Here, we present the total number of neocortical neurons in two strains of pigs. In the adult Göttingen minipig, the neocortex contains 324 million neurons, whereas the domestic pig brain contains 432 million neocortical neurons. This 33% strain difference in neocortical neuron number was not an unexpected finding considering the general relationship between body size and neuron number (for a review, see Williams and Herrup, 1988). The total number of neocortical neurons has also been estimated in a number of other species. There are ~3 million neocortical neurons in the mouse brain (Bonthius et al., 2004), 21 million in the rat (Korbo et al., 1990), 12.8 billion in the minke whale (Nina Eriksen and Bente Pakkenberg, unpublished data) and 19–23 billion in the human neocortex (Pakkenberg and Gundersen, 1997). When compared to these species, one of the most valuable findings in the pig brain is the rather low coefficient of variation (CV) from which the true biological variance can be estimated to be less than 10% (see the additivity of variances below). The low biological variance seems to be a reproducible finding for the pig brain (Holm and West, 1994; Larsen et al., 2004; Jelsing et al., 2005a; Jelsing et al., 2005b) and supports the continued use of pigs in neurotoxicologic studies, since perturbations may be detected with great sensitivity.

Stereological design

Estimates of neocortical cell numbers were obtained using the optical fractionator method, which is efficient and independent of any tissue shrinkage or expansion that may take place during any stage of tissue preparation (Gundersen, 1986; West et al., 1991). It was considered optimal on paraffin sections in which shrinkage during processing is significant. Provided that mounted sections maintain a sufficient depth to accommodate optical disectors, the optical fractionator can also be applied on other preparations, e.g. cryostat or vibratome sections.

Sampling parameters were optimized to obtain a high efficiency in terms of precision and effort. A total count of ~100–150 cells in 75–100 disectors distributed systematically randomly on 5–10 sections is usually enough to obtain an estimate with a precision appropriate for most biological structures (Pakkenberg and Gundersen, 1988; Korbo et al., 1990; West, 1993). However, because the variance in the experimental groups appeared to be rather low, it was decided to increase the efficiency of the fractionator by intensifying the sampling of cells to an average of 250 particles per brain in 140–170 disectors. An additional number of sections were sampled from the Göttingen minipig. This was done to ensure enough sections for forthcoming studies of specific glial subpopulations, of which some have a more heterogeneous distribution in the brain. The efficiency of the fractionator sampling was evaluated from variance analysis of relative variances and estimator CE. These two measures are related through the basic equation (the additivity of variances):

$$CV^2_{obs} = CV^2_{biol} + CE^2 . \quad (6)$$

The sampling was considered optimal when the variance of the individual estimate, CE^2, was less than half the observed interindividual variance, CV^2, because the real inherent biological variability in the cohorts of pigs (CV^2_{biol}) then contributes most to the observed relative variance. Subsequently, the estimator CE was optimized in relation to variances from sampling with disectors within a section and sampling between sections (Table 3).

Even though the efficiency and mathematical unbiasedness of the optical fractionator method for estimating cells in the pig brain neocortex are evident, some fundamental

requirements have to be fulfilled for a proper application (West, 1993). A first requirement is that the whole structure is accessible; secondly, one must be confident that all cells of interest can be identified unambiguously and that penetration of staining is complete throughout the thickness of the section. In the present study, a complete penetration of staining was optimized beforehand by registering the z-distribution of all cells. However, difficulty in distinguishing glial cells from neurons appeared when stained with the modified Giemsa method. This was especially true in the neonate brains, where the neuronal density was high and morphometric differences between cells were less pronounced. The lack of clear criteria for distinguishing neurons and glial cells in cortical regions has previously been a major problem for stereologists (Braendgaard et al., 1990; Davanlou and Smith, 2004) and may partly explain the somewhat lower hemispheric cell number published in a screening procedure of young Göttingen minipig (Jelsing et al., 2005a). Recently, a number of immunohistochemical markers have been evaluated in the pig brain (Lyck et al., 2006), and the combination of immunohistochemistry and stereology may provide a better approach for quantifying neurons and glial cell populations in future quantitative studies of the pig brain.

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