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Developmental Changes in Expression of $GABA_A$ Receptor Subunits α_1, α_2, and α_3 in the Pig Brain

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Abstract
GABA is a major neurotransmitter in the mammalian brain. In the mature brain GABA exerts inhibitory actions via the $GABA_A$ receptor ($GABA_AR$); however, in the immature brain GABA provides much of the excitatory drive. We examined the expression of 3 predominant $GABA_A$ α-subunit proteins in the pig brain at various pre- and postnatal ages. Brain tissue was collected from piglets born via caesarean section at preterm ages 91, 97, 100, and 104 days' gestational age (GA), at term equivalent (114 days' GA, caesarean section) and at term, postnatal day 0 (P0) (spontaneous delivery, term = 115 days). Tissue was obtained from piglets at P4 and P7. Adult tissue from sows was collected postmortem after caesarean section. In all cortical regions and basal ganglia (1) α_3 exhibited a significant increase in protein expression at 100 days' GA, (2) α_3 expression decreased with age after 100 days' GA, (3) α_1 increased with age, with peak expression at P7 in cortices, hippocampus, and thalamus, and (4) α_2 protein expression remained relatively constant across the ages examined. The subunit expression of α_3 was most abundant at preterm ages, with α_1 the predominant subunit expressed postnatally. Immunofluorescent labelling revealed α_1 expression on the somatic membranes of pyramidal cells in the cortex and hippocampus, and in the cerebellar Purkinje cells. Positive α_3 labelling was apparent on interneurones in the cortex and hippocampus. The switch between dominant α-subunits may coincide with the functional change in GABAergic neurotransmission from excitation to inhibition. Brain growth in the pig closely reflects that in the term human, making the pig a valuable non-primate model for studying development and the effects of insults on the perinatal brain.

Introduction

In the mature brain the $GABA_A$ receptor ($GABA_AR$) mediates fast inhibitory synaptic transmission [1]. In the immature brain GABA contributes to the excitatory drive of developing cortical networks [2, 3] with the transition in function from excitation to inhibition during the course of brain maturation. In the rat this is proposed to occur at roughly term-equivalent age of postnatal days P10–P12 [4]. In humans, however, this functional switch may not be complete until 4 months' postnatal age [5]. While the function of the $GABA_AR$ is driven by the ex-

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pression of the cation chloride ion (Cl^-) co-transporters, which are responsible for maintaining the intracellular Cl^- concentration, it has been suggested that the presence of specific α-subunits may also contribute to the maturation of the $GABA_AR$ inhibitory function [6].

The $GABA_AR$ is a pentameric Cl^- channel composed of several subunits. In the mammalian brain various isoforms of each subunit have been identified; namely α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, and θ [7]. Different subunit co-assembly results in various receptor subtypes, with differing pharmacological functions [7–9]. In the adult brain the most abundant subtypes of $GABA_AR$ contain an α_1, α_3, or α_2 isoform in combination with a γ_2 subunit and either a β_2 or β_3 to form the majority of functional $GABA_AR$ [10]. GABA is the first neurotransmitter active in the mammalian brain and plays a critical role in regulating cortical development [2, 9, 11] with reported expression of functional $GABA_ARs$ on cortical neural stem cells [12–15]. The composition and expression of early life $GABA_ARs$, however, is different from those expressed in later life and likely corresponds to the developmental changes in GABAergic activity. mRNA studies in the neonatal rat brain have revealed that of the $GABA_AR$ α-subunits, the α_3 and α_2 subunits predominate in the developing brain, and it is not until around birth that the α_1 subunit increases in expression and significantly dominates in the adult brain [16].

While extensive research on the effects of pre- and postnatal factors on the neonatal brain have utilised rodent models, the rodent brain develops on a substantially different time course and is lissencephalic compared with larger mammals, such as pigs. Therefore the piglet can provide a research model wherein results are more translatable to humans, and use of the neonatal piglet in neuroscientific research is increasing [17–19]. The pig is an appealing research model because, like humans, the major brain growth spurt extends from the late prenatal to the postnatal period [20, 21]. Gross anatomical features, including the presence of gyri and sulci and the distribution of grey and white matter are similar to those of a human neonate [17, 21, 22].

In this study we used Western blot analysis to determine the expression of α_1, α_2, and α_3 $GABA_AR$ subunits in the frontal, parietal, temporal, and occipital cortices, basal ganglia, hippocampus, thalamus, and cerebellum at 9 time points ranging from prenatal to adult. We then demonstrated the cellular localisation using immunofluorescence techniques. We showed a significant cortical upregulation of the α_1 subunit at P7 in cortical regions, with a pattern of increased α_1 expression with increasing age observed in non-cortical regions. The α_3 subunit showed a unique peak of expression at 100 days' gestational age (GA) (85% gestation) in all regions except the cerebellum, followed by a rapid decline at 104 days' GA, and then plateauing of expression. Immunofluorescent studies co-labelling for the α_1 and α_3 subunits were performed. The pattern of staining changed with age, with the α_3 subunit observed to be located predominantly on interneurone subtypes in the cortex, hippocampus, and cerebellum. α_1 labelling was evident at all ages, with limited labelling at earlier preterm ages in the cortex, cerebellum, and hippocampus proper. α_1 labelling was evident on somatic membranes and dendrites, particularly in the cerebellar Purkinje cells and hippocampal pyramidal cells.

Table 1. Data on body weight and whole brain weight (cerebrum, cerebellum, and brain stem) for the animals

Age, days	Body weight, kg	Brain weight, g	M:F
91d	0.74±0.13	19.16±1.06	3:2
97d	0.90±0.10	21.94±2.84	2:3
100d	0.94±0.20	24.16±3.19	1:4
104d	1.12±0.17	27.44±2.04	2:3
114d	1.91±0.53	36.08±2.43	1:4
P0	1.44±0.24	32.14±2.49	3:2
P4	1.96±0.22	35.44±1.48	2:3
P7	2.70±0.28	39.84±3.41	3:2

Values are mean ± SD unless otherwise indicated. Age: 91d, 97d, 100d, 104d, and 114d indicate gestational age in days; P0, P4, and P7 indicate postnatal age in days.

Materials and Methods

Animals and Tissue Preparation

Approval for this study was granted by the University of Queensland Animal Ethics Committee and carried out in accordance with the National Health and Medical Research Council guidelines (NHMRC, Australia). Five Large White piglets from each of the following postnatal ages were obtained from The University of Queensland Gatton Piggery at term (P0, day of birth), P4, and P7. Piglets were weighed and euthanised with an intracardiac injection of sodium pentobarbital (325 mg/kg). Preterm and term-equivalent piglets (n = 5 per age group: term = 115 days' gestation, such that preterm = 91, 97, 100, and 104, and term-equivalent = 114 days' gestation) were obtained by caesarean section. Data on body weight and whole brain weight (cerebrum, cerebellum, and brain stem) for the animals are summarised in Table 1.

Pregnant sows were initially anaesthetised with azaperone (0.7 mg/kg, i.v.) and alfaxalone (1.2 mg/kg, i.v.) and maintained with

isoflurane anaesthesia (1–2% in air) for the duration of the surgery [23]. Upon delivery, the piglets were weighed and euthanised with an injection of sodium pentobarbital (325 mg/kg, intracardiac or umbilical artery). Sows were euthanised with an intravenous injection of sodium pentobarbital (325 mg/kg).

The whole brain was removed from all piglets and 5 of the sows and sectioned into coronal slices of 3–4 mm. The brain was hemisected, the right hemisphere slices were immersion-fixed overnight in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) with agitation at room temperature, and then stored at 4°C in 0.1% paraformaldehyde until paraffin-embedding and subsequent microtome sectioning.

Regions of interest including 4 cortical regions, basal ganglia, hippocampus, thalamus, and cerebellum, were dissected from the left hemisphere and slow frozen in 0.32 M sucrose at –80°C [24] until Western blot analysis.

Western Blot Analysis

Frozen tissue was weighed and homogenised in 10× volume of ice-cold ddH_2O. Following homogenisation, samples were centrifuged at 1,400 *g* for 5 min at 4°C. The supernatant was collected and protein concentration determined by bicinchoninic acid assay as per the manufacturer's instructions (BCA; Pierce, Thermo Fisher Scientific, Scoresby, VIC, Australia). Total crude protein homogenates were combined with 5× sample buffer (10% SDS, 30% glycerol, 5% β-mercaptoethanol, 50 mM Tris, pH 6.8) and boiled for 5 min (100°C). Ten micrograms of total protein from each sample was electrophoresed on 10% sodium dodecyl sulphate-polyacrylamide gel. A pooled protein standard, generated by combining all samples, was run on every gel (5, 10, and 30 μg) to control for variability in gel electrophoresis conditions, transfer efficiency, and final quantification purposes [25].

Following electrophoresis and transfer to polyvinylidene fluoride membrane (Immobilon PVDF; Merck-Millipore Australia Pty Ltd., Bayswater, VIC, Australia), membranes were blocked for 1 h in 1% low-fat skim milk powder in Tris-buffered saline (TBST, 140 mM NaCl, 2 mM KCl, 25 mM Tris, 0.1% Tween-20 v/v) with agitation at room temperature. Primary antibodies were added to the blocking solution, and membranes were incubated overnight at 4°C on an orbital shaker. Membranes were incubated with rabbit polyclonal anti-$GABA_AR$ α_1 (1:3,000; #AB5609), α_2 1:3,000; #AB5948), and α_3 (1:15,000; #AB5594) commercial antibodies (Merck-Millipore, Australia). The membranes were washed 3 × 10 min in TBST, and secondary goat anti-rabbit IgG-peroxidase antibody (#A0545, Sigma-Aldrich, St. Louis, MO, USA) was applied at 1:30,000 for 1 h at room temperature in 1% skim milk/TBST. The membranes were washed as above, then incubated with enhanced chemiluminescence reagent (Luminata Forte; Merck-Millipore), and proteins visualised on X-ray film. Protein expression was quantitated by densitometry analysis with Image-J software (National Institutes of Health, Bethesda, MD, USA), and the relative level of protein expression was determined from the standard curve generated from pooled standard samples on each blot [25].

Immunofluorescence

Paraffin-embedded tissue sections (8 μm) containing parietal cortex, hippocampus, and cerebellum were immunolabelled (n = 3 per age group). Tissue was dewaxed and rehydrated through xylenes and graded alcohols, using an automated system (Leica ST5010 Autostainer XL). Antigen retrieval was performed using Diva Decloaker, pH 6.0 at 100°C for 20 min (Biocare Medical, distributed by MetaGene, Manly, QLD, Australia). Sections were washed twice for 10 min in 0.1 M PBS with 0.05% Triton X-100 v/v (PBST) and blocked in 10% bovine serum albumin (BSA-PBST) for 30 min. Sections were rinsed in PBST and incubated overnight in 5% BSA with primary antibodies; mouse monoclonal anti-$GABA_A$ α_1 (1:6,000; #MAB339; Merck-Millipore) and polyclonal rabbit anti-α_3 (1:500; #AB5594; Merck-Millipore) in a humidified chamber. Sections were washed 3 × 10 min in PBST and species-specific secondary antibodies applied (1:500, donkey anti-mouse IgG Alexa Fluor 488; goat anti-rabbit IgG Alexa Fluor 568; Invitrogen, Life Technologies, Waverley, VIC, Australia) in 1% BSA-PBST for 1.5 h in a dark humidified chamber. Sections were washed twice in PBS and once in PBST for 10 min, and coverslipped with Prolong Gold with 4',6-diamidino-2-phenylindole (DAPI) as a nuclear stain (Invitrogen Life Technologies). Images of immunolabelled sections were acquired using an Olympus microscope (BX41) and photographed using a CCD camera (Olympus DP70; Olympus Pty Ltd., Australia). Secondary only (negative) controls were also run to rule out non-specific binding (data not shown).

Statistical Analysis

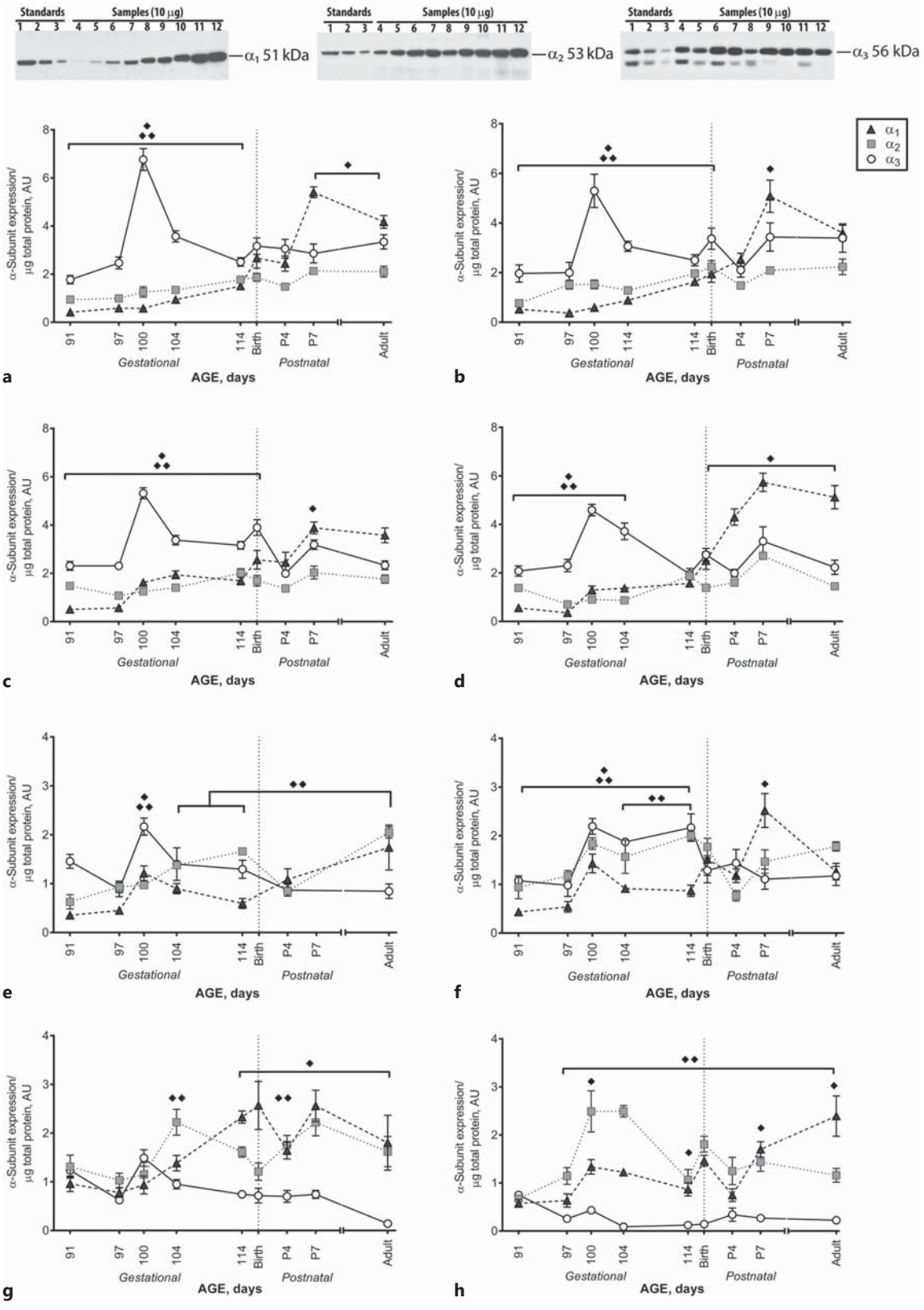
The Levene test for equal variances and the Shapiro-Wilk test for normality of data were performed; data was not normally distributed, and subsequently ranked. Kruskal-Wallis non-parametric ANOVA was used to determine differences in each α-subunit protein expression across age groups. Pairwise comparisons were used to determine differences between α-subunit expression within each age group, with $p < 0.05$ considered significant (SPSS Statistics 22.0; IBM Corporation, Armonk, NY, USA). Graphs represent mean protein expression at each age with standard error bars (GraphPad Prism 6.0 Software; La Jolla, CA, USA).

Results

Western Blot Analysis

A single immunoreactive band was observed and quantitated for $GABA_AR$ α_1 (at 51 kDa) and α_2 (at 53 kDa). Two bands were observed for α_3 but in the current study only the upper band at 56 kDa was quantitated (Fig. 1); this smaller band may result from post-translational modification, although this usually results in larger molecular weight products, or it could be another α-isoform [26]. Significant changes were observed in α_1 and α_3 subunit protein expression in the age groups studied; however, the α_2 protein retained relatively stable expression across cortical development.

There was significantly higher α_3 expression in the cortical regions at the preterm ages investigated compared with α_1; this was reversed postnatally such that α_1 became the predominantly expressed α-subunit isoform in the cortices by P7 (Fig. 1a–d). In the non-cortical regions α_3 expression was significantly higher than α_1 in the hippocampus at 104 and 114 days; after this time point there



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was a downregulation in α_3 expression. The cerebellum exhibited the lowest α_3 expression, with α_1 expression significantly higher at all ages from 97 days onwards (Fig. 1h). In the thalamus and cerebellum α_2 expression was higher than α_1 and α_3 prenatally, and remained more highly expressed than α_3 at all ages investigated.

Across the developmental period investigated regional changes in the upregulation of α_1 were observed. In cortical regions α_1 expression markedly increased postnatally, whereas in non-cortical regions increased expression was observed as early as 100 days. Higher α_1 expression was also observed in the cortices compared with other brain regions, with basal ganglia α_1 expression significantly lower than frontal, temporal, and occipital cortices. The highest level of α_1 protein expression was observed in the frontal and occipital cortices at P7. The α_1 isoform showed a marked upregulation in protein expression in all cortical regions, and a smaller but still significant upregulation in the non-cortical regions.

In all brain regions, except the cerebellum, a peak in α_3 expression was observed at 100 days. The most significant increase in α_3 expression was observed in the frontal cortex, with a 4-fold increase in protein expression compared with expression at 97 days (Fig. 1a). Cortical expression of α_3 subunit decreased by 104 days and tended to plateau to levels observed at earlier preterm ages. In the non-cortical regions α_3 expression continued to decrease postnatally to levels of expression lower than observed prenatally. Significantly higher cortical α_3 expression was observed when compared with the non-cortical regions at any given age.

Subunit expression of α_2 either remained constant or exhibited a small upregulation in protein expression across development. A significant upregulation in α_2 expression was observed in the frontal and parietal cortices and basal ganglia by adulthood. In the cerebellum this upregulation occurred at around 100–104 days (Fig. 1h); expression levels of the α_2 subunit were then observed to decrease at 114 days to levels similar to those observed prior to 100 days.

Immunofluorescence Results

For relative expression levels of $GABA_AR$ α_1 and α_3 subunit expression in the cortical, hippocampal, and cerebellar cell layers, refer to Table 2. Across various brain regions α_1 expression increased with age, whereas marked reductions in α_3 expression were observed in the cortex and cerebellum.

Frontal Cortex

The α_1 immunofluorescence revealed differences in laminar distribution across development. At preterm ages there appears to be more diffuse weak immunoreactivity of α_1 throughout the cortical layers (Fig. 2, 91d and 100d). Staining on the membrane around the soma and on dendrites of some pyramidal cells in layer V was evident at the earliest time point studied. By 114 days there is intense neuropil labelling evident in layer III and IV. At P7 and adult there is distinctive neuropil staining in layer II that was not strongly apparent at earlier ages. Upregulation of α_1 immunoreactivity was observed in the outer and inner pyramidal cell layers and inner granular layer (layers III–V) in the adult brain.

The α_3 immunofluorescence also revealed differences in the cellular and laminar distribution across development. At preterm ages prominent α_3 staining was observed in the superficial cortical layers with evident localisation to stellate cells (layer I–II), and strong immunoreactivity was also observed in the polymorphic layer (layer VI) and subplate. By P0 and P4 α_3 labelling was more restricted to layer IV and layer VI. At higher magnification it was observed that α_3 appeared to be on cell

Fig. 1. Representative Western Blots of α_1, α_2, and α_3 subunits. Lanes 1–4 pooled standard samples (30, 20, 10, 5 μg). Lanes 5–12 temporal cortex samples (10 μg) from 91, 97, 100, 104, and 114 days' gestation, P0, P4, P7, and adult. Graphs represent changes in α-subunit protein expression across development: α_1 (triangles), α_2 (squares), α_3 (circles); mean with standard error bars, $p < 0.05$. AU, arbitrary units. **a** Frontal cortex α_3 was significantly higher than α_1 (91–114 days); α_1 was significantly higher than α_2 and α_3 (P7 and adult). **b** Parietal cortex α_3 was significantly higher than α_1 (91–114 days); α_1 was significantly higher than α_2 (P4–P7). **c** Temporal cortex α_3 was significantly higher than α_1 (91–114 days) and significantly higher than α_2 (100 and 104 days and P0); α_1 was significantly higher than α_2 (P7). **d** Occipital cortex α_3 was significantly higher than α_1 (91–100 days), significantly higher than α_2 (91 days–P0); α_1 was significantly higher than α_2 (P0 – adult) and significantly higher than α_3 (P7 and adult). **e** Basal ganglia α_3 was significantly higher than α_1 (91–100 days) and significantly higher than α_2 (100 days); α_2 was significantly higher than α_1 (104 and 114 days) and significantly higher than α_3 (adult). **f** Hippocampus α_3 was significantly higher than α_1 (91–114 days); α_2 was significantly higher than α_1 (104 and 114 days); α_1 was significantly higher than α_3 (P7). **g** Thalamus α_2 was significantly higher than α_3 (104 days, P4); α_1 was significantly higher than α_3 (114 days–adult). **h** Cerebellum α_2 was significantly higher than α_3 (97 days–adult, except at P4) and significantly higher than α_1 (97 days); α_1 was significantly higher than α_3 (100 and 114 days, P7, and adult).

Table 2. Relative expression levels of $GABA_AR$ α_1 and α_3 subunit expression in the cortical, hippocampal, and cerebellar cell layers

	$GABA_AR$ α_1									$GABA_AR$ α_3								
	91d	97d	100d	104d	114d	P0	P4	P7	adult	91d	97d	100d	104d	114d	P0	P4	P7	adult
Cerebral cortex																		
Layer I	+	+/–	+/–	+	+/–	+/–	+/–	+	+	++	+	++	++	++	++	+	++	++
Layer II (preterm II/III)	+/–	+/–	+/–	+	+/–	–	–	+/–	+	+	+/–	++	++	+/–	+/–	+/–	+	+
Layer III					+/–	+	+/–	+/–	+					+	+	+/–	+	+
Layer IV(preterm IV/V)	+/–	+	+/–	+	+	+	+	+	++	+/–	+/–	++	+	+	+/–	+	+/–	+
Layer V					++	++	++	++	+++					+/–	+	+/–	+	+/–
Layer VI	+	+	+/–	+	+	+	+	+/–	+	+++	+++	+++	+++	+++	+++	++	+	++
Hippocampus (CA1)																		
Stratum oriens	+	+	+	++	+	++	+++	++	+++	+	+	++	++	+	+	++	++	++
Stratum pyramidale	+	+	+	++	++	+	++	++	++	+/–	+	+	++	+	+/–	+	++	+
Stratum radiatum	+	+	+	+	+	++	++	++	++	+/–	+	+	++	++	++	++	++	++
Stratum moleculare	++	++	+	+	+	+	++	++	++	–	–	+	+/–	+/–	+	+	+/–	+
Stratum granulosum	+	+/–	+/–	+/–	+/–	+/–	+	+	+	+	+/–	+	+	+	+/–	+	+	+
Hippocampal hilus	++	++	++	++	++	++	+++	+++	+++	+	+	++	++	++	+	+	+	+
Cerebellum																		
Molecular layer	++	++	+++	+++	++	++	++	++	++	+	+	+	+	+	+	+	+	+
Purkinje cell layer	+	+	+	+	+/–	+/–	+	+	–	+	+/–	+/–	+	+	+/–	+	+	+
Granular layer	+/–	+/–	+	+	+++	+++	++	+++	+++	++	+	+	+	+	+	+/–	+/–	+

Age: 91d, 97d, 100d, 104d, and 114d indicate gestational age in days; P0, P4, and P7 indicate postnatal age in days. Protein expression from α_1 and α_3 immunofluorescence labelling was assessed as intense; +++, strong positive; ++, positive; +, weakly positive; +/–, immunoreactivity not detectable.

types discretely different from α_1-positive cells: α_1 immunoreactivity appeared distinctly on some pyramidal cells, whereas α_3 labelling appeared confined to interneurones and stellate cells, with occasional punctate α_3 labelling apparent on the nuclear membrane of some pyramidal cells (Fig. 2, P4).

Hippocampus

Positive immunoreactivity of α_1 was evident on some hippocampal pyramidal cells, on the somatic membrane, and on both apical and basal dendrites in CA1 from as early as 91 days (Fig. 3). A substantial increase in the number of α_1 positive pyramidal cells was observed at P0 and P4.

Positive α_3 immunoreactivity was evident on basket cells in the stratum oriens and radiatum in the CA1 region at all ages, with upregulation at 100 and 104 days' gestation. Strong α_3 immunoreactivity was observed at 100 and 104 days in the pyramidal cell layer (Fig. 3, 100d). Punctate α_3 labelling was visible on the nuclear membrane of some hippocampal hilar cells, with staining on the membrane of the soma and processes of basket cells located in the hippocampal hilus.

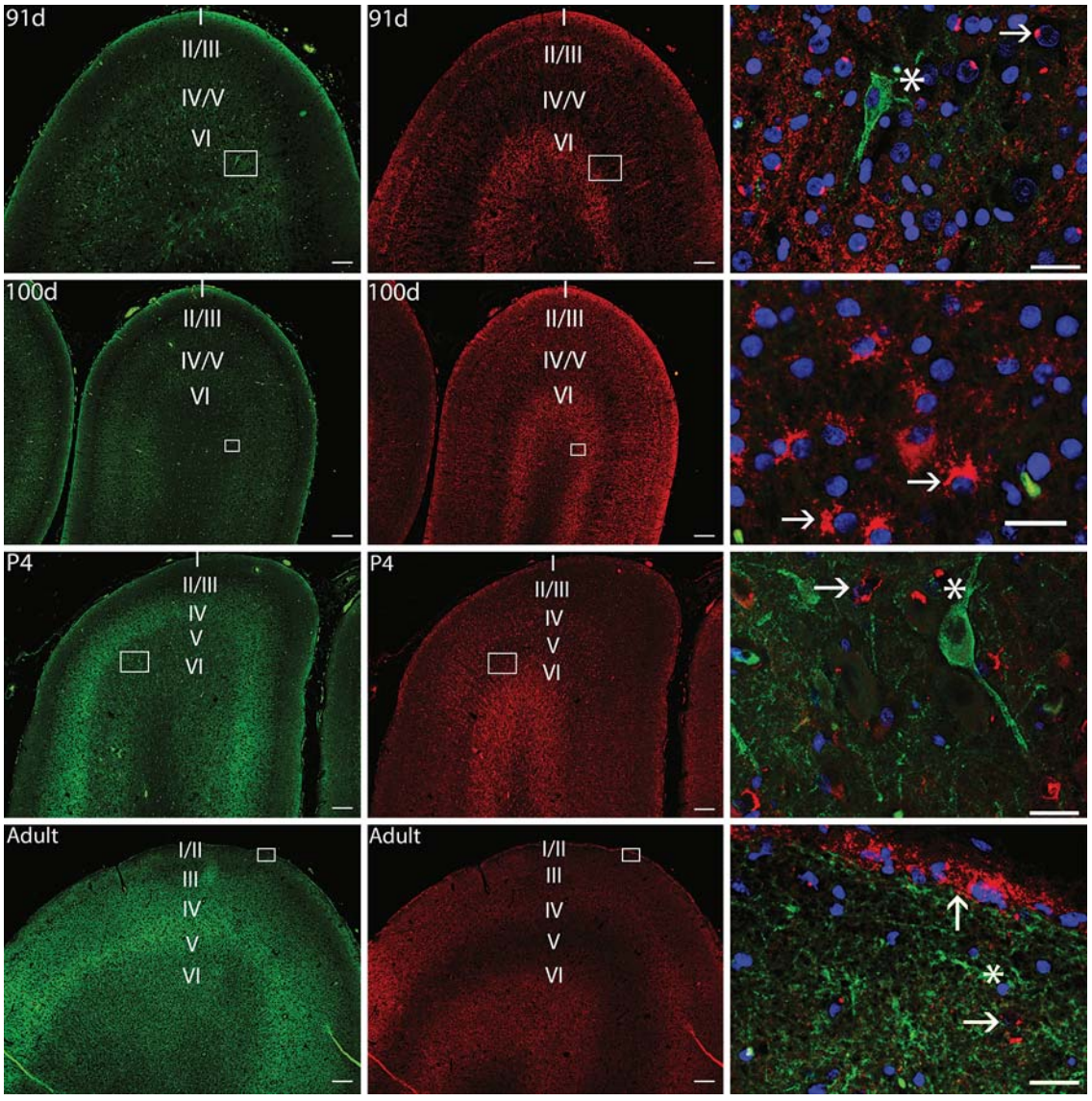
At higher magnification, the α_3 labelling of pyramidal cells appeared to be restricted to the soma, whereas α_1 labelling was distinctly evident on the membrane of the soma and processes, visible from the earliest age investigated of 91 days. As with cortical staining, α_1 and α_3 appeared on discretely different cells, and did not appear to co-localise.

Cerebellum

Strong labelling of α_1 was apparent from 97 days on the dendritic arbour of the molecular layer, with labelling evident at the earlier age of 91 days. Labelling on the somatic membrane of the Purkinje cells was also visible at all ages investigated. In the granular layer strong α_1 labelling was apparent from 114 days through to adulthood, with weak labelling seen at preterm ages (Fig. 4).

Strong α_3 labelling was seen at preterm ages in the granular layer on presumed Golgi cells (based on soma size and granular layer location). By 114 days this α_3 labelling was less evident, with fewer positively labelled cells. Stellate cells in the molecular layer were immunoreactive for α_3 at all ages, with staining of the soma and processes evident.

At higher magnification, α_1 and α_3 appeared to co-label Purkinje cells at the earliest age of 91 days' gestation, although by 97 days there appeared to be discrete α_1 labelling of the somatic membrane of Purkinje cells. Punctate α_3 labelling was visible on the nuclear membrane of some cerebellar Purkinje cells and basket cells at all ages.



Color version available online

Fig. 2. $GABA_A$ α_1 (green) and α_3 (red) labelling with DAPI nuclear stain (blue) in frontal cortex (magnification as specified) (colors refer to the online version only). 91d, 91 days' gestation: at 10× magnification, with α_1 and α_3 labelling in layer VI at higher magnification (40×). 100d, 100 days' gestation: at 4× magnification, with α_3 labelling in layer VI at higher magnification (40×). P4: at 4× magnification, with discrete α_1 and α_3 labelling in layer IV–V at higher magnification (40×). Adult: at 4× magnification, with α_3 labelling on the pial surface, with α_1 neuropil labelling in layer I–II at 40×. Asterisks indicate α_1 positive immunolabelling, arrows indicate α_3 positive immunolabelling. Scale bars: at 4×, 250 μm; 10×, 100 μm; 40×, 25 μm.

Immunofluorescent labelling with $GABA_AR$ α_2 antibodies was unsuccessful after trialling 2 separate antibodies directed toward the N-terminus (#SC-7350; Santa Cruz, TX, USA) and the C-terminus (#AB5948; Merck-Millipore, Australia). No detectable fluorescence was observed under various antigen retrieval conditions and antibody dilutions.

Discussion

The pig is an excellent model for human brain growth [18, 21, 22] and is frequently used in preclinical research models of neonatal hypoxic brain injury and treatment [27–32]. The major brain growth spurt of the pig occurs in the late prenatal to postnatal period as in humans, and

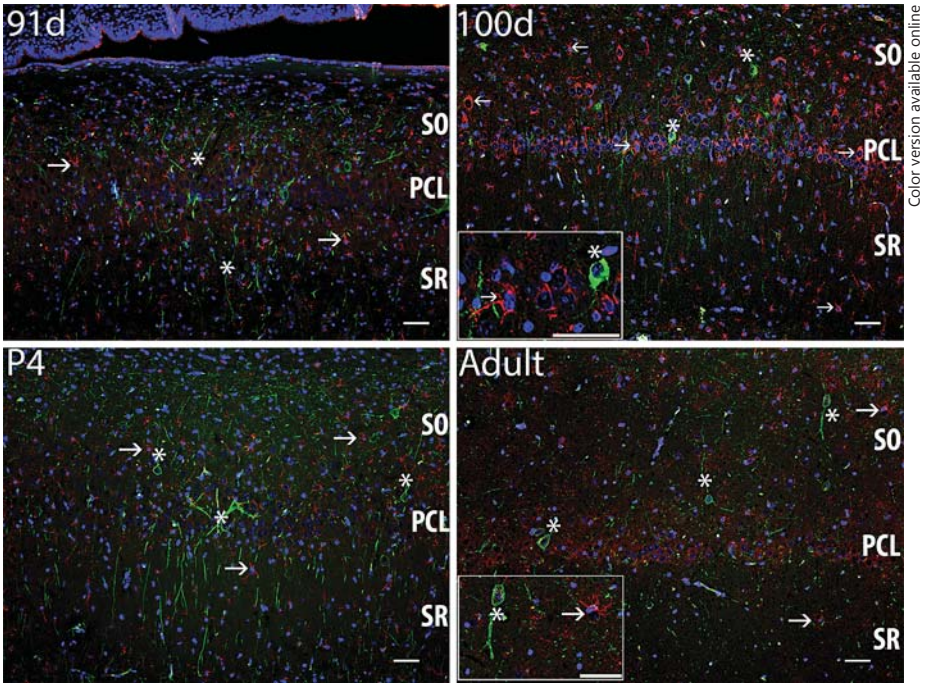


Fig. 3. $GABA_A$ α_1 (green) and α_3 (red) labelling with DAPI nuclear stain (blue) in hippocampus at 20× magnification (colors refer to the online version only). SO, stratum oriens; PCL, pyramidal cell layer; SR, stratum radiatum. 91d, 91 days' gestation; 100d, 100 days' gestation, with **inset** showing pyramidal cell positively labelled with α_1 and adjacent cells positively labelled with α_3; P4; adult, with **inset** showing α_3 positively labelled basket cell. Asterisks indicate α_1 positive immunolabelling, arrows indicate α_3 positive immunolabelling. Scale bars, 50 μm.

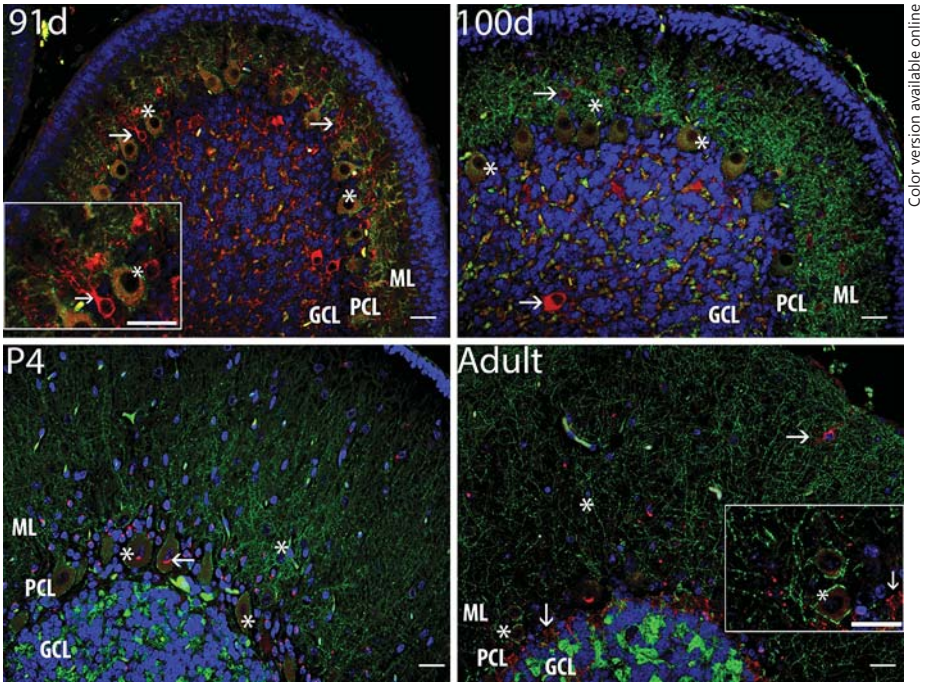


Fig. 4. $GABA_A$ α_1 (green) and α_3 (red) labelling with DAPI nuclear stain (blue) in cerebellum at 40× magnification (colors refer to the online version only). GCL, granule cell layer; PCL, Purkinje cell layer; ML, molecular layer. 91d, 91 days' gestation, with **inset** showing weak α_1 labelling on membrane of Purkinje cell and α_3 labelling of Golgi cell; 100d, 100 days' gestation; P4; adult, with **inset** showing distinct α_1 labelling on Purkinje cell membranes with adjacent discrete α_3 labelling. Asterisks indicate α_1 positive immunolabelling, arrows indicate α_3 positive immunolabelling. Scale bars, 25 μm.

is characterised by glial cell multiplication, dendrite growth, synapse formation, and myelination [20, 22, 33]. Pond et al. [22] expanded on the pioneering work of Dobbing and colleagues [20, 33] by including analysis of total protein, cholesterol, and DNA content, as well as brain weight, of piglets at pre- and postnatal ages . When assessing brain weight relative to previous time points, the authors noted a biphasic brain growth spurt pattern in the Large White pig brain. Pond et al. [22] postulated that this could be due to initial neuronal multiplication and maturation, followed by glial cell multiplication.

In our study the crossover of dominant subunit expression from α_3 to α_1 varied regionally and temporally, intersecting earliest in the cerebellum, at around (and possibly prior to) 91 days (80% gestation in pig). The thalamus also had a prenatal change in the dominant expression of the α_1 subunit prior to 97 days. In all other brain regions these changes occurred around the time of birth or postnatally, with the latest switch occurring in the frontal cortex at around P4. By P7 in all regions assessed, α_1 was the predominant subunit, with the limitation that there were no basal ganglia samples available for this time point.

The mammalian brain goes through a period of peak growth during development; however, the timing of this peak varies widely across species. In some larger mammals such as sheep and monkey, this happens prior to birth, while in rodents and rabbits peak brain growth is not observed until some days to weeks postnatally [20, 22]. So finding the appropriate model to investigate developmental changes in the GABAergic system can be challenging. In the pig, however, peak brain growth has been shown to occur around birth, similar to that of the term human neonate [22, 33].

The gyrencephalic piglet brain is increasingly being recognised as a useful non-primate model for human brain developmental studies, with myelination [34] and brain composition [22, 35] similar to the human brain. Jelsing et al. [21] assessed neuronal populations in the domestic pig and concluded that the domestic pig should be used preferentially for studies investigating development and the effects of perinatal insults on the human brain [18, 19, 21].

The results from the present study in the piglet brain provide some valuable insights into the region-specific differences in the timing of the developmental switch in $GABA_AR$ α-isoforms that has been reported to occur sometime around birth in rats and humans [36, 37]. In the brain regions examined, data from the present study showed that (1) α_3 was the predominant α-subunit expressed during prenatal cortical and hippocampal development, and (2) α_1 expression was relatively low during prenatal development, but increased after birth to become the dominant α-subunit in the cortices, thalamus, and cerebellum in the mature brain. This "switch" in α-subunit dominance, is in concordance with previously published mRNA studies in the rat brain [16, 38], the human brain [5], and with immunohistochemistry results [4, 39, 40].

We observed no significant downregulation in α_2 expression with age, as has been previously observed in rodent studies [16]. In our observations α_2 was moderately lowly expressed at all ages, and protein expression levels remained relatively constant across development. We observed higher α_2 expression in the non-cortical regions studied compared with cortical expression. This concurs with previously reported data in the neonatal and adult rat showing that α_2 is strongly expressed in the hippocampus, amygdala, hypothalamus, and thalamus [16, 41]. Higher α_2 mRNA expression in the hippocampus, compared with the frontal cortex, has previously been reported by Wisden et al. [42] in the bovine brain, with only weak expression of α_1 and α_3 in the pyramidal cell layer.

Apart from the significant peak in expression at 100 days of the α_3 subunit, both the α_3 and α_2 subunit expression levels remained consistent across cortical development through to adulthood, while the α_1 protein significantly increased. In the non-cortical regions examined α_2 expression fluctuated with levels of expression, with a significant prenatal peak in expression observed in the thalamus and cerebellum. Similar to cortical development, α_1 expression continued to increase with age in the non-cortical regions.

Immunofluorescence studies concurred with the Western blot analysis: in the cortex, hippocampus, and cerebellum both α_1 and α_3 were visibly detectable at all ages. The cellular distribution of the α_1 and α_3 subunits varied, with strong cytosolic labelling for α_3 on pyramidal cells of the hippocampus at 100 and 104 days' gestation, whereas α_1 appeared membrane-bound on the soma and processes of discretely different pyramidal cells. The positive labelling of α_1 on *excitatory* cell types, cortical, hippocampal pyramidal, and cerebellar granule cells [43, 44], would imply that the presence of the α_1 subunit could be associated with the inhibitory feedback systems of cell excitation. The localisation of α_1 to the cell membrane suggests that this subunit is likely to be present in membrane-bound $GABA_AR$ complexes. Cortical expression of α_1 and α_3 in the adult matched what has previously been reported by Wisden et al. [42] in the bovine brain, with strong α_1 immunoreactivity in layers II–IV, and stronger α_3 labelling in the deeper layers V–VI.

A novel finding in this study is the marked increase in protein expression of the α_3 subunit at 100 days in all regions examined except the cerebellum. Further investigations into the cellular distribution of the α_3 subunit using immunofluorescence revealed an upregulation of α_3 expression in the soma of pyramidal cells and an increase in the number of α_3 immunoreactive basket cells in the hippocampus. A similar increase in α_3 immunoreactivity cortical stellate cells was also observed. In this study we

observed a peak in α_3 subunit expression that appears to coincide with a prenatal growth spurt in brain weight as evidenced by an increase in the brain:body weight ratio (see Eiby et al., 2013, Fig. 2) [23].

Conclusion

This paper describes the developmental changes in protein expression of the $GABA_AR$ α-subunit during development in the pig brain – documented as a model more similar to the human particularly with regard to the timing of the brain growth spurt [20, 22, 33]. Results from our current study show a significant upregulation of α_1 subunit expression that may coincide with the switch in function of the $GABA_AR$ from excitatory to inhibitory that is driven by the Cl^- co-transporters NKCC1 and KCC2 [15, 40, 45, 46]. While the development of the $GABA_AR$ has been extensively investigated in the rat brain [4, 16, 38, 39, 41, 47, 48], the value of larger animals as preclinical models for the study of human disease has gained much interest. Further investigations into the functional capacity (through binding studies) of the α-subunits investigated here would broaden our understanding of the temporal and regional development of the $GABA_AR$ system in the pig brain.

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Disclosure Statement

The authors report no conflicts of interest.

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