

# Developmental Changes in Expression of GABA<sub>A</sub> Receptor Subunits alfa1, alfa2, dan alfa3 in the Pig Brain

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# Developmental Changes in Expression of GABA<sub>A</sub> Receptor Subunits $\alpha_1$ , $\alpha_2$ , and $\alpha_3$ in the Pig Brain

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## Keywords

Neonate · Perinatal brain · Neurotransmission

## Abstract

GABA is a major neurotransmitter in the mammalian brain. In the mature brain GABA exerts inhibitory actions via the GABA<sub>A</sub> receptor (GABA<sub>A</sub>R); however, in the immature brain GABA provides much of the excitatory drive. We examined the expression of 3 predominant GABA<sub>A</sub>  $\alpha$ -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)  $\alpha_3$  exhibited a significant increase in protein expression at 100 days' GA, (2)  $\alpha_3$  expression decreased with age after 100 days' GA, (3)  $\alpha_1$  increased with age, with peak expression at P7 in cortices, hippocampus, and thalamus, and (4)  $\alpha_2$  protein expression remained relatively constant across the ages examined. The subunit expression of  $\alpha_3$  was most abundant at preterm ages, with  $\alpha_1$  the predominant subunit expressed postna-

tally. Immunofluorescent labelling revealed  $\alpha_1$  expression on the somatic membranes of pyramidal cells in the cortex and hippocampus, and in the cerebellar Purkinje cells. Positive  $\alpha_3$  labelling was apparent on interneurons in the cortex and hippocampus. The switch between dominant  $\alpha$ -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.

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## Introduction

In the mature brain the GABA<sub>A</sub> receptor (GABA<sub>A</sub>R) 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<sub>A</sub>R is driven by the ex-

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

The  $\text{GABA}_A\text{R}$  is a pentameric  $\text{Cl}^-$  channel composed of several subunits. In the mammalian brain various isoforms of each subunit have been identified; namely  $\alpha_{1-6}$ ,  $\beta_{1-3}$ ,  $\gamma_{1-3}$ ,  $\delta$ ,  $\epsilon$ ,  $\pi$ , and  $\theta$  [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  $\text{GABA}_A\text{R}$  contain an  $\alpha_1$ ,  $\alpha_3$ , or  $\alpha_2$  isoform in combination with a  $\gamma_2$  subunit and either a  $\beta_2$  or  $\beta_3$  to form the majority of functional  $\text{GABA}_A\text{R}$  [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  $\text{GABA}_A\text{Rs}$  on cortical neural stem cells [12–15]. The composition and expression of early life  $\text{GABA}_A\text{Rs}$ , 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  $\text{GABA}_A\text{R}$   $\alpha$ -subunits, the  $\alpha_3$  and  $\alpha_2$  subunits predominate in the developing brain, and it is not until around birth that the  $\alpha_1$  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  $\alpha_1$ ,  $\alpha_2$ , and  $\alpha_3$   $\text{GABA}_A\text{R}$  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  $\alpha_1$  subunit at P7 in cortical regions, with a pattern of increased  $\alpha_1$  expression with increasing

**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.

age observed in non-cortical regions. The  $\alpha_3$  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  $\alpha_1$  and  $\alpha_3$  subunits were performed. The pattern of staining changed with age, with the  $\alpha_3$  subunit observed to be located predominantly on interneurone subtypes in the cortex, hippocampus, and cerebellum.  $\alpha_1$  labelling was evident at all ages, with limited labelling at earlier preterm ages in the cortex, cerebellum, and hippocampus proper.  $\alpha_1$  labelling was evident on somatic membranes and dendrites, particularly in the cerebellar Purkinje cells and hippocampal pyramidal cells.

## 56 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. 25

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<sub>2</sub>O. 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<sub>A</sub>R α<sub>1</sub> (1:3,000; #AB5609), α<sub>2</sub> (1:3,000; #AB5948), and α<sub>3</sub> (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<sub>A</sub> α<sub>1</sub> (1:6,000; #MAB339; Merck-Millipore) and polyclonal rabbit anti-α<sub>3</sub> (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

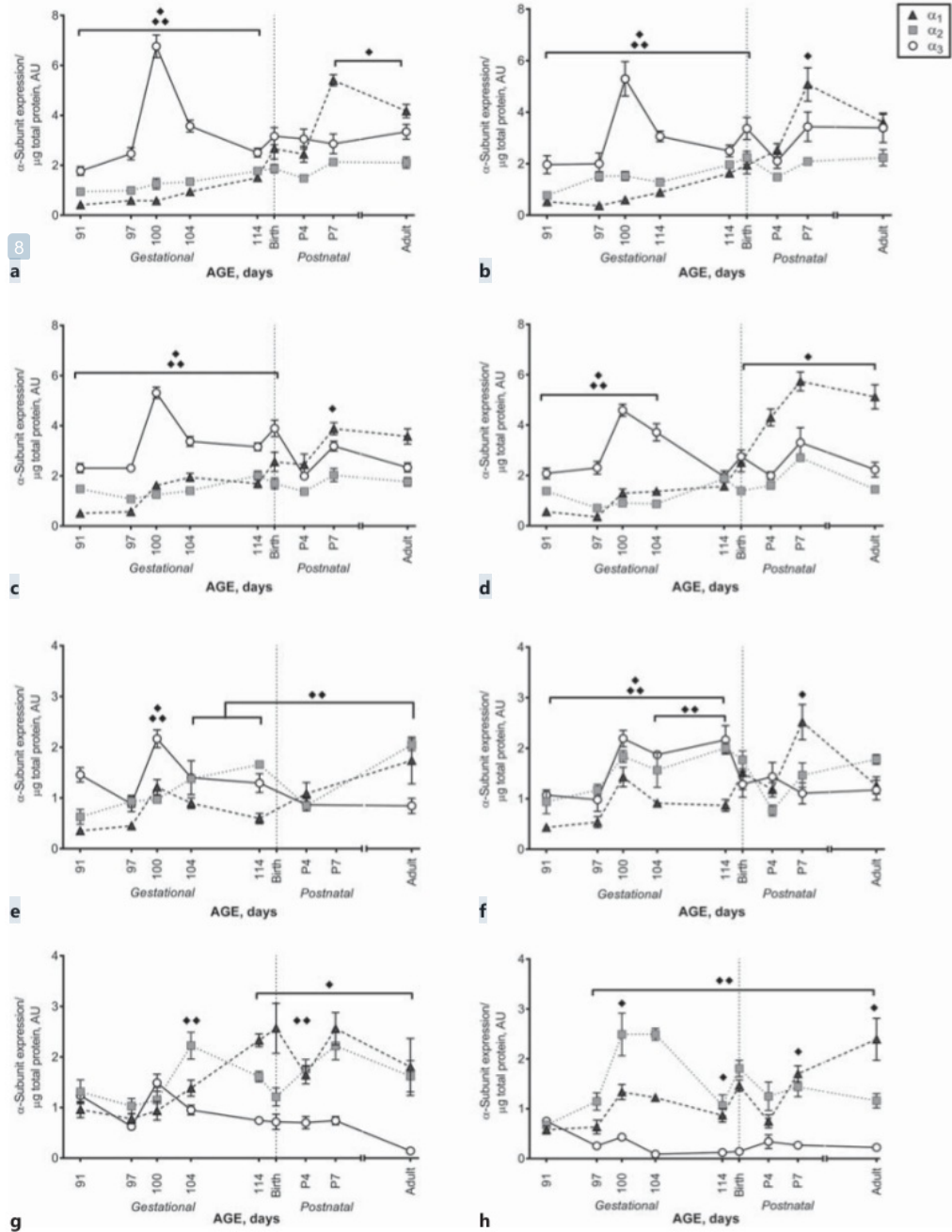
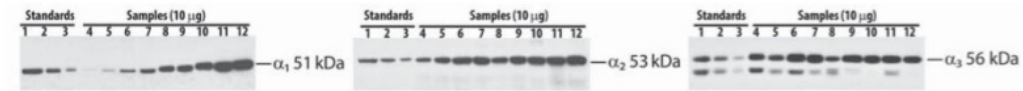
The Levene test for equal variances and the Shapiro-Wilk test for normality of data were performed; data was not normally distributed, and subsequently ranked. Kruskal-Wallis non-parametric ANOVA was used to determine differences in each α-subunit expression across age groups. Pairwise comparisons were 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<sub>A</sub>R α<sub>1</sub> (at 51 kDa) and α<sub>2</sub> (at 53 kDa). Two bands were observed for α<sub>3</sub> 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<sub>1</sub> and α<sub>3</sub> subunit protein expression in the age groups studied; however, the α<sub>2</sub> protein retained relatively stable expression across cortical development.

There was significantly higher α<sub>3</sub> expression in the cortical regions at the preterm ages investigated compared with α<sub>1</sub>; this was reversed postnatally such that α<sub>1</sub> became the predominantly expressed α-subunit isoform in the cortices by P7 (Fig. 1a–d). In the non-cortical regions α<sub>3</sub> expression was significantly higher than α<sub>1</sub> in the hippocampus at 104 and 114 days; after this time point there



(For legend see next page.)

1

was a downregulation in  $\alpha_3$  expression. The cerebellum exhibited the lowest  $\alpha_3$  expression, with  $\alpha_1$  expression significantly higher at all ages from 97 days onwards (Fig. 1h). In the thalamus and cerebellum  $\alpha_2$  expression was higher than  $\alpha_1$  and  $\alpha_3$  prenatally, and remained more highly expressed than  $\alpha_3$  at all ages investigated.

Across the developmental period investigated regional changes in the upregulation of  $\alpha_1$  were observed. In cortical regions  $\alpha_1$  expression markedly increased postnatally, whereas in non-cortical regions increased expression was observed as early as 100 days. Higher  $\alpha_1$  expression was also observed in the cortices compared with other brain regions, with basal ganglia  $\alpha_1$  expression significantly lower than frontal, temporal, and occipital cortices. The highest level of  $\alpha_1$  protein expression was observed in the frontal and occipital cortices at P7. The  $\alpha_1$  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  $\alpha_3$  expression was observed at 100 days. The most significant increase in  $\alpha_3$  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  $\alpha_3$  subunit decreased by 104 days and tended to plateau to levels observed at earlier preterm ages. In the non-cortical regions  $\alpha_3$  expression continued to decrease postnatally to levels of expression lower than observed prenatally. Significantly higher cortical  $\alpha_3$  expression was observed when compared with the non-cortical regions at any given age.

Subunit expression of  $\alpha_2$  either remained constant or exhibited a small upregulation in protein expression across development. A significant upregulation in  $\alpha_2$  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  $\alpha_2$  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<sub>A</sub>R  $\alpha_1$  and  $\alpha_3$  subunit expression in the cortical, hippocampal, and cerebellar cell layers, refer to Table 2. Across various brain regions  $\alpha_1$  expression increased with age, whereas marked reductions in  $\alpha_3$  expression were observed in the cortex and cerebellum.

#### Frontal Cortex

The  $\alpha_1$  immunofluorescence revealed differences in laminar distribution across development. At preterm ages there appears to be more diffuse weak immunoreactivity of  $\alpha_1$  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  $\alpha_1$  immunoreactivity was observed in the outer and inner pyramidal cell layers and inner granular layer (layers III–V) in the adult brain.

The  $\alpha_3$  immunofluorescence also revealed differences in the cellular and laminar distribution across development. At preterm ages prominent  $\alpha_3$  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  $\alpha_3$  labelling was more restricted to layer IV and layer VI. At higher magnification it was observed that  $\alpha_3$  appeared to be on cell

**Fig. 1.** Representative Western Blots of  $\alpha_1$ ,  $\alpha_2$ , and  $\alpha_3$  subunits. Lanes 1–4 pooled standard samples (30, 20, 10, 5  $\mu$ g). Lanes 5–12 temporal cortex samples (10  $\mu$ g) from 91, 97, 100, 104, and 114 days' gestation, P0, P4, P7, and adult. Graphs represent changes in  $\alpha$ -subunit protein expression across development:  $\alpha_1$  (triangles),  $\alpha_2$  (squares),  $\alpha_3$  (circles); mean with standard error bars,  $p < 0.05$ . AU, arbitrary units. **a** Frontal cortex  $\alpha_3$  was significantly higher than  $\alpha_1$  (91–114 days);  $\alpha_1$  was significantly higher than  $\alpha_2$  and  $\alpha_3$  (P7 and adult). **b** Parietal cortex  $\alpha_3$  was significantly higher than  $\alpha_1$  (91–114 days);  $\alpha_1$  was significantly higher than  $\alpha_2$  (P4–P7). **c** Temporal cortex  $\alpha_3$  was significantly higher than  $\alpha_1$  (91–114 days) and significantly higher than  $\alpha_2$  (100 and 104 days and P0);  $\alpha_1$  was significantly higher than  $\alpha_2$  (P7). **d** Occipital cortex  $\alpha_3$  was signifi-

cantly higher than  $\alpha_1$  (91–100 days), significantly higher than  $\alpha_2$  (91 days–P0);  $\alpha_1$  was significantly higher than  $\alpha_2$  (P0 – adult) and significantly higher than  $\alpha_3$  (P7 and adult). **e** Basal ganglia  $\alpha_3$  was significantly higher than  $\alpha_1$  (91–100 days) and significantly higher than  $\alpha_2$  (100 days);  $\alpha_2$  was significantly higher than  $\alpha_1$  (104 and 114 days) and significantly higher than  $\alpha_3$  (adult). **f** Hippocampus  $\alpha_3$  was significantly higher than  $\alpha_1$  (91–114 days);  $\alpha_2$  was significantly higher than  $\alpha_1$  (104 and 114 days);  $\alpha_1$  was significantly higher than  $\alpha_3$  (P7). **g** Thalamus  $\alpha_2$  was significantly higher than  $\alpha_3$  (104 days, P4);  $\alpha_1$  was significantly higher than  $\alpha_3$  (114 days–adult). **h** Cerebellum  $\alpha_2$  was significantly higher than  $\alpha_3$  (97 days–adult, except at P4) and significantly higher than  $\alpha_1$  (97 days);  $\alpha_1$  was significantly higher than  $\alpha_3$  (100 and 114 days, P7, and adult).

**Table 2.** Relative expression levels of GABA<sub>A</sub>R  $\alpha_1$  and  $\alpha_3$  subunit expression in the cortical, hippocampal, and cerebellar cell layers

	GABA <sub>A</sub> R $\alpha_1$									GABA <sub>A</sub> R $\alpha_3$								
	91d	97d	100d	104d	114d	P0	P4	P7	adult	91d	97d	100d	104d	114d	P0	P4	P7	adult
<i>Cerebral cortex</i>																		
Layer I	±	+/-	+/-	+	+/-	+/-	+/-	+	+	++	+	++	++	++	++	+	++	++
Layer II (preterm II/III)	+/-	+/-	+/-	+	+/-	-	-	+/-	+	+	+/-	++	++	+/-	+/-	+/-	+	+
Layer III					+/-	+	+/-	+/-	+					+	+	+/-	+	+
Layer IV (preterm IV/V)	+/-	+	+/-	+	+	+	+	+	++	+/-	+/-	++	+	+	+/-	+	+/-	+
Layer V					++	++	++	++	+++					+/-	+	+/-	+	+/-
Layer VI	+	+	+/-	+	+	+	+	+/-	+	+++	+++	+++	+++	+++	+++	++	+	++
<i>Hippocampus (CA1)</i>																		
Stratum oriens	+	+	+	++	+	++	+++	++	+++	+	+	++	++	+	+	++	++	++
Stratum pyramidale	+	+	+	++	++	+	++	++	++	+/-	+	+	++	+	+/-	+	++	+
Stratum radiatum	+	+	+	+	+	++	++	++	++	+/-	+	+	++	++	++	++	++	++
Stratum moleculare	++	++	+	+	+	+	++	++	++	-	-	+	+/-	+/-	+	+	+/-	+
Stratum granulosum	+	+/-	+/-	+/-	+/-	+/-	+	+	+	+	+/-	+	+	+	+/-	+	+	+
Hippocampal hilus	++	++	++	++	++	++	+++	+++	+++	+	+	++	++	++	+	+	+	+
<i>Cerebellum</i>																		
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  $\alpha_1$  and  $\alpha_3$  immunofluorescence labelling was assessed as intense; +++, strong positive; ++, positive; +, weakly positive; +/-, immunoreactivity not detectable.

types discretely different from  $\alpha_1$ -positive cells:  $\alpha_1$  immunoreactivity appeared distinctly on some pyramidal cells, whereas  $\alpha_3$  labelling appeared confined to interneurons and stellate cells, with occasional punctate  $\alpha_3$  labelling apparent on the nuclear membrane of some pyramidal cells (Fig. 2, P4).

### Hippocampus

Positive immunoreactivity of  $\alpha_1$  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  $\alpha_1$  positive pyramidal cells was observed at P0 and P4.

Positive  $\alpha_3$  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  $\alpha_3$  immunoreactivity was observed at 100 and 104 days in the pyramidal cell layer (Fig. 3, 100d). Punctate  $\alpha_3$  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  $\alpha_3$  labelling of pyramidal cells appeared to be restricted to the soma, whereas  $\alpha_1$  labelling was distinctly evident on the membrane of the

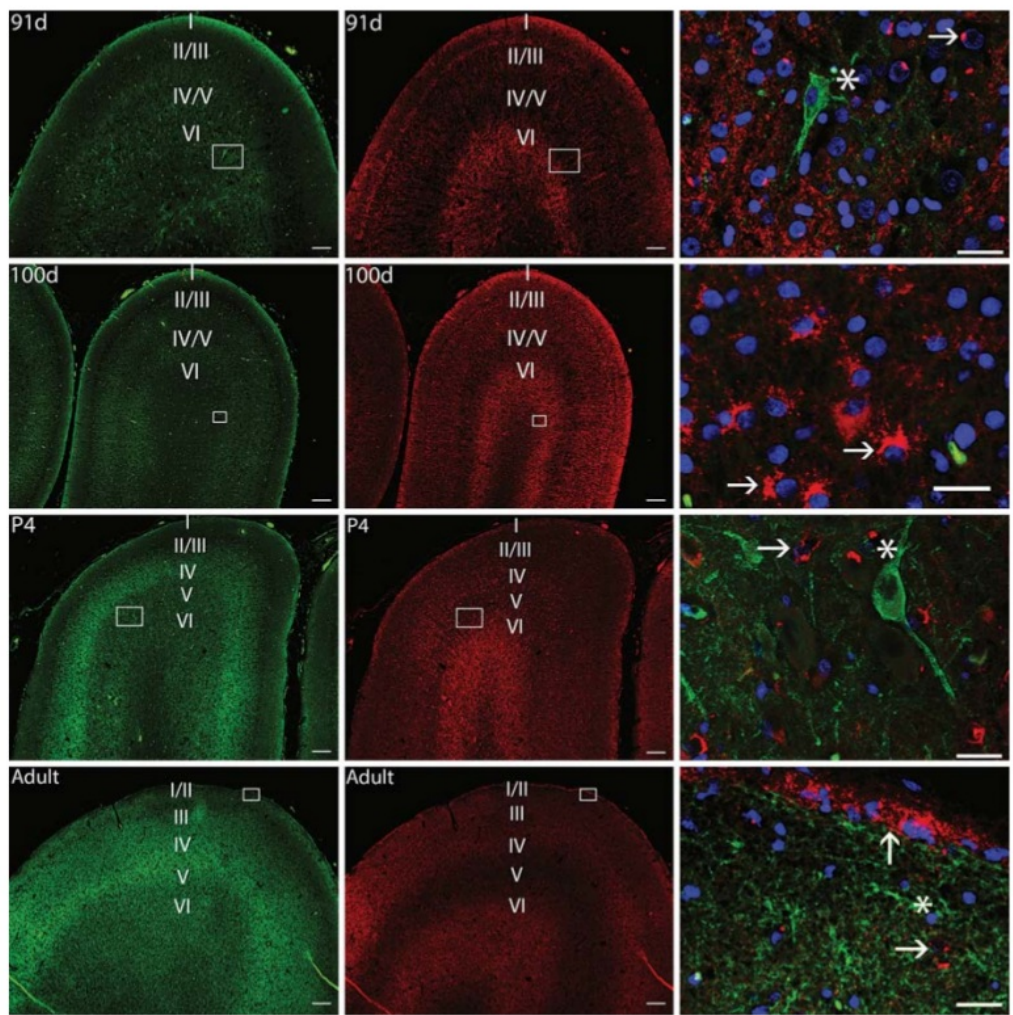
soma and processes, visible from the earliest age investigated of 91 days. As with cortical staining,  $\alpha_1$  and  $\alpha_3$  appeared on discretely different cells, and did not appear to co-localise.

### Cerebellum

Strong labelling of  $\alpha_1$  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  $\alpha_1$  labelling was apparent from 114 days through to adulthood, with weak labelling seen at preterm ages (Fig. 4).

Strong  $\alpha_3$  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  $\alpha_3$  labelling was less evident, with fewer positively labelled cells. Stellate cells in the molecular layer were immunoreactive for  $\alpha_3$  at all ages, with staining of the soma and processes evident.

At higher magnification,  $\alpha_1$  and  $\alpha_3$  appeared to co-label Purkinje cells at the earliest age of 91 days' gestation, although by 97 days there appeared to be discrete  $\alpha_1$  labelling of the somatic membrane of Purkinje cells. Punctate  $\alpha_3$  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<sub>A</sub> α<sub>1</sub> (green) and α<sub>3</sub> (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<sub>1</sub> and α<sub>3</sub> labelling in layer VI at higher magnification (40×). 100d, 100 days' gestation: at 4× magnification, with α<sub>3</sub> labelling in layer VI at higher magnification (40×). P4: at

4× magnification, with discrete α<sub>1</sub> and α<sub>3</sub> labelling in layer IV–V at higher magnification (40×). Adult: at 4× magnification, with α<sub>3</sub> labelling on the pial surface, with α<sub>1</sub> neuropil labelling in layer I–II at 40×. Asterisks indicate α<sub>1</sub> positive immunolabelling, arrows indicate α<sub>3</sub> positive immunolabelling. Scale bars: at 4×, 250 μm; 10×, 100 μm; 40×, 25 μm.

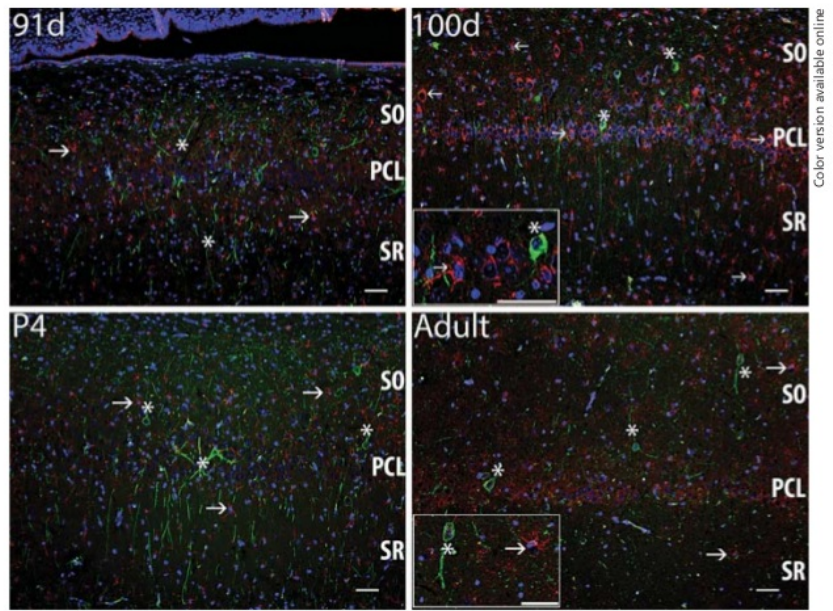
Immunofluorescent labelling with GABA<sub>A</sub> α<sub>2</sub> antibodies was unsuccessful after trialling 2 separate antibodies directed toward the N-terminus (#SC-7350; Santa Cruz, TX, USA) and the C-terminus (#AB5948; Merck-Millipore, Australia). No detectable fluorescence was observed under various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

## Discussion

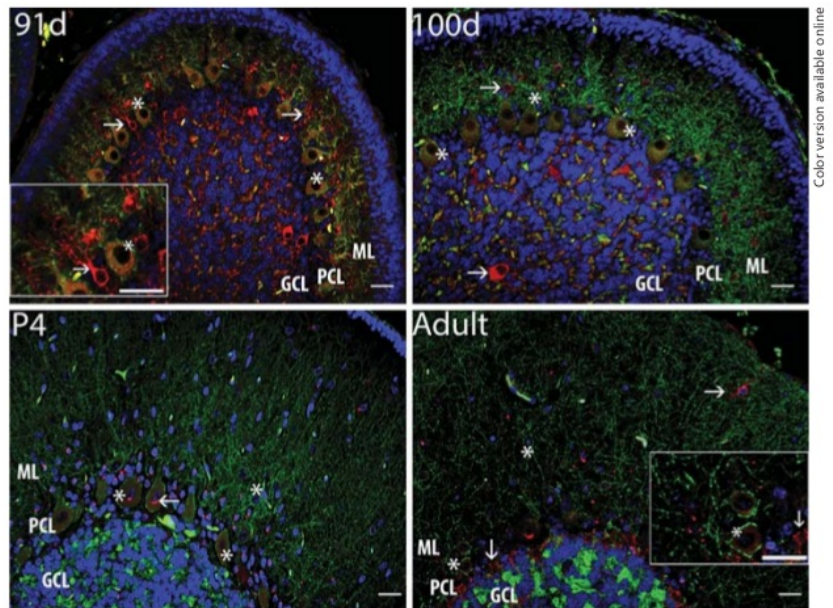
The pig is an excellent model for human brain growth [18, 21, 22] and is frequently used in preclinical research models of neonatal hypoxic brain injury and treatment [27–32]. The major brain growth spurt of the pig occurs in the late prenatal to postnatal period as in humans, and



**Fig. 3.** GABA<sub>A</sub>  $\alpha_1$  (green) and  $\alpha_3$  (red) labelling with DAPI nuclear stain (blue) in hippocampus at 20 $\times$  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  $\alpha_1$  and adjacent cells positively labelled with  $\alpha_3$ ; P4; adult, with **inset** showing  $\alpha_3$  positively labelled basket cell. Asterisks indicate  $\alpha_1$  positive immunolabelling, arrows indicate  $\alpha_3$  positive immunolabelling. Scale bars, 50  $\mu$ m.



**Fig. 4.** GABA<sub>A</sub>  $\alpha_1$  (green) and  $\alpha_3$  (red) labelling with DAPI nuclear stain (blue) in cerebellum at 40 $\times$  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  $\alpha_1$  labelling on membrane of Purkinje cell and  $\alpha_3$  labelling of Golgi cell; 100d, 100 days' gestation; P4; adult, with **inset** showing distinct  $\alpha_1$  labelling on Purkinje cell membranes with adjacent discrete  $\alpha_3$  labelling. Asterisks indicate  $\alpha_1$  positive immunolabelling, arrows indicate  $\alpha_3$  positive immunolabelling. Scale bars, 25  $\mu$ m.



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 assess-

ing 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  $\alpha_3$  to  $\alpha_1$  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  $\alpha_1$  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,  $\alpha_1$  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<sub>A</sub>R  $\alpha$ -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)  $\alpha_3$  was the predominant  $\alpha$ -subunit expressed during prenatal cortical and hippocampal development, and (2)  $\alpha_1$  expression was relatively low during prenatal development, but increased after birth to become the dominant  $\alpha$ -subunit in the cortices, thalamus, and cerebellum in the mature brain. This “switch” in  $\alpha$ -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  $\alpha_2$  expression with age, as has been previously observed in ro-

dent studies [16]. In our observations  $\alpha_2$  was moderately lowly expressed at all ages, and protein expression levels remained relatively constant across development. We observed higher  $\alpha_2$  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  $\alpha_2$  is strongly expressed in the hippocampus, amygdala, hypothalamus, and thalamus [16, 41]. Higher  $\alpha_2$  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  $\alpha_1$  and  $\alpha_3$  in the pyramidal cell layer.

Apart from the significant peak in expression at 100 days of the  $\alpha_3$  subunit, both the  $\alpha_3$  and  $\alpha_2$  subunit expression levels remained consistent across cortical development through to adulthood, while the  $\alpha_1$  protein significantly increased. In the non-cortical regions examined  $\alpha_2$  expression fluctuated with levels of expression, with a significant prenatal peak in expression observed in the thalamus and cerebellum. Similar to cortical development,  $\alpha_1$  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  $\alpha_1$  and  $\alpha_3$  were visibly detectable at all ages. The cellular distribution of the  $\alpha_1$  and  $\alpha_3$  subunits varied, with strong cytosolic labelling for  $\alpha_3$  on pyramidal cells of the hippocampus at 100 and 104 days' gestation, whereas  $\alpha_1$  appeared membrane-bound on the soma and processes of discretely different pyramidal cells. The positive labelling of  $\alpha_1$  on *excitatory* cell types, cortical, hippocampal pyramidal, and cerebellar granule cells [43, 44], would imply that the presence of the  $\alpha_1$  subunit could be associated with the inhibitory feedback systems of cell excitation. The localisation of  $\alpha_1$  to the cell membrane suggests that this subunit is likely to be present in membrane-bound GABA<sub>A</sub>R complexes. Cortical expression of  $\alpha_1$  and  $\alpha_3$  in the adult matched what has previously been reported by Wisden et al. [42] in the bovine brain, with strong  $\alpha_1$  immunoreactivity in layers II–IV, and stronger  $\alpha_3$  labelling in the deeper layers V–VI.

A novel finding in this study is the marked increase in protein expression of the  $\alpha_3$  subunit at 100 days in all regions examined except the cerebellum. Further investigations into the cellular distribution of the  $\alpha_3$  subunit using immunofluorescence revealed an upregulation of  $\alpha_3$  expression in the soma of pyramidal cells and an increase in the number of  $\alpha_3$  immunoreactive basket cells in the hippocampus. A similar increase in  $\alpha_3$  immunoreactivity cortical stellate cells was also observed. In this study we

41 observed a peak in  $\alpha_3$  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

40 This paper describes the developmental changes in protein expression of the GABA<sub>A</sub>  $\alpha$ -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  $\alpha_1$  subunit expression that may coincide with the switch in function of the GABA<sub>A</sub> receptor from excitatory to inhibitory that is driven by the Cl<sup>-</sup> co-transporters NKCC1 and KCC2 [15, 40, 45, 46]. While the development of the GABA<sub>A</sub> receptor 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  $\alpha$ -

subunits investigated here would broaden our understanding of the temporal and regional development of the GABA<sub>A</sub> receptor system in the pig brain.

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## 12 Disclosure Statement

The authors report no conflicts of interest.

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PAGE 1

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PAGE 2

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PAGE 3

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PAGE 4

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PAGE 5

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PAGE 6

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PAGE 7

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PAGE 8

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PAGE 9

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PAGE 10

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PAGE 11

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