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$GABA_A$ receptor expression and white matter disruption in intrauterine growth restricted piglets

by

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Highlights

- $GABA_A$ receptor α subunit expression is altered in the IUGR piglet brain
- White matter disruption evident in IUGR at multiple gestational time-points
- $GABA_A$ receptor may be a key factor in white matter injury in the IUGR brain
- Altered GABA system may contribute to cognitive disabilities observed in IUGR
- Understanding mechanisms of IUGR brain injury essential to identifying treatments

Abstract

Intrauterine growth restriction (IUGR) is one of the most common causes of perinatal mortality and morbidity. White matter and neuronal injury are major pathophysiological features of the IUGR neonatal brain. $GABA_A$ (γ-aminobutyric acid type A) receptors have been shown to play a role in oligodendrocyte differentiation and proliferation in the neonatal brain and may be a key factor in white matter injury and myelination in IUGR neonates. Whether there are impairments to the GABAergic system and neuronal cytoskeleton in IUGR brain has yet to be elucidated. This study aims to examine $GABA_A$ receptor α_1 and α_3 subunit protein expression and distribution in parietal cortex and hippocampus of the IUGR piglet at four different ages (term = 115d - days gestational age), 100d, 104d, birth (postnatal day 0 – P0) and P7 and to examine neuronal and myelination patterns. Significant alterations to $GABA_A$ receptor α_1 and α_3 protein expression levels were observed in the IUGR piglet brain of P7 IUGR piglets with significantly greater α_3 expression compared to α_1 expression in the hippocampus while there was virtually no difference between the two subunits in the parietal cortex. However a significantly lower α_1/α_3 ratio was evident in P7 IUGR cortex when compared with P7 NG cortex. Neuronal somatodendrites studied using MAP2 immunohistochemistry showed reduced and disrupted somatodendrites while MBP immunolabelling showed loss of axonal fibres from gestational day 104d through to P7. These findings provide insights into the effects of IUGR on the development of the GABA system, altered developmental maturation of $GABA_A$ receptor subunit expression in the IUGR brain may influence myelination and may partly explain the cognitive disabilities observed in IUGR. Understanding the mechanisms behind grey and white matter injury in the IUGR infant is essential to identifying targets for treatments to improve long-term outcomes for IUGR infants.

Keywords

GABA, neuronal injury, myelin, low birth weight, placental insufficiency.

1. Introduction

Intrauterine growth restriction (IUGR) is one of the most common causes of perinatal mortality and morbidity (Allen, 2001; Gillespie, 2002). In developing countries a high prevalence of IUGR is reported with affected infants more likely to develop learning and cognitive impairments later in life (Allen, 2001; De Bie, 2010; Gillespie, 2002). Hypoxemia and impaired nutrient supply during pregnancy are major contributors to brain pathophysiology in IUGR neonates (Cox and Marton, 2009; Economides DL, 1989; Nicolaides KH, 1989).

White matter and neuronal injury are major pathophysiological features of the IUGR neonatal brain. Clinical imaging studies demonstrate alterations in brain structure in IUGR infants including altered white and grey matter volumes, decreased levels of brain connectivity, decreased cortical thickness and delayed cortical development (Esteban et al., 2010; Padilla et al., 2015; Tolsa et al., 2004). These alterations that persist at 1 year of age are associated with developmental disabilities in the IUGR infant (Esteban et al., 2010; Padilla et al., 2011).

Myelination of axons, which is critical for effective neuronal communication begins shortly after birth and progresses into adulthood (Kinney et al., 1994). Myelination involves a series of signals between oligodendrocytes and neurons involving several neurotransmitters and growth factors. Adenosine, glutamate, GABA (γ-aminobutyric acid), and ATP are able to modulate oligodendrocyte progenitor proliferation, differentiation, and migration along with oligodendrocyte survival and myelination (Arellano et al., 2016). In particular, $GABA_A$ receptors have been shown to play a role in oligodendrocyte differentiation and proliferation in the neonatal brain (Zonouzi et al., 2015). GABAergic signaling regulates oligodendrocyte progenitor cell differentiation and proliferation in the preterm neonatal brain and may be a key factor in diffuse white matter injury in these neonates (Zonouzi et al., 2015).

GABA is a major neurotransmitter in the neonatal brain. $GABA_A$ receptors are shown to have a neurotrophic action complementary to their neurotransmission function, with a role in neuronal cell migration in cortical layers (Doris and Arnold, 2009; Lujan et al., 2005). $GABA_A$ receptor subunits are regulated in a distinctive spatial and temporal manner, both during development and into adulthood in the brain (Brooks-Kayal and Pritchett, 1993; Chen et al., 2001; Hornung and Fritschy, 1996; Laurie et al., 1992; Montpied et al., 1989; Poulter et al., 1992; Takayama and Inoue, 2004). Cerebral function depends on adequate development

of essential inhibitory neural circuits and the appropriate amount of excitation and inhibition at specific stages of maturation. Early prenatal neuronal synaptic responses to GABA are initially excitatory however during the early postnatal period in the rat, $GABA_A$ receptor responses switch to inhibitory (Ben-Ari, 2002; Plotkin et al., 1997). We have previously demonstrated the change in $GABA_A$ receptor α_3 to α_1 subunit expression at birth in the normal developing piglet brain (Kalanjati et al., 2011) coinciding with the excitatory/inhibitory switch. The timing of the switch in $GABA_A$ receptor function in humans however has not been firmly established and may not be complete until 3-4 months of age (Herlenius and Lagercrantz, 2001; Murphy et al., 2005). Changes in $GABA_A$ receptor subunit expression alter the receptor composition and modify $GABA_A$ receptor function and thus GABA neurotransmission (Puia et al., 1991; Sieghart and Sperk, 2002; Verdoorn et al., 1990). The α_1 subunit-containing $GABA_A$ receptor has a higher affinity for GABA with a faster activation and deactivation period compared to the α_3 subunit-containing receptor (Keramidas and Harrison, 2010; Verdoorn, 1994; Verdoorn et al., 1990) .

Normal growth of neurons, neuronal cytoskeletons and myelination are vital for synaptogenesis (Stafstrom, 2007). Together, with the evolving balance of the GABA system, they play a key role in normal brain maturation and activity (Stafstrom, 2007). In animal models of IUGR using diet restriction, vascular ligation or placental embolization, impaired GABA, neuronal somatodendritic growth and myelination were observed (Steiger et al., 2003; Tashima et al., 2001). However, limited research has involved animal models with naturally occurring IUGR. Unlike the induction of IUGR through surgery or drugs necessary in small animal models, IUGR occurs spontaneously in the pig and therefore serves as an excellent pre-clinical model.

This study aims to elucidate the ontogeny of the $GABA_A$ receptor α_1 and α_3 subunit protein expression and distribution patterns in IUGR piglet parietal cortex and hippocampus at four different age points, 100 days gestational age (~30wk human GA), 104 days gestational age (>32wk human GA), birth (P0) and post-natal 7 days (P7) and to examine neuronal and myelination patterns in IUGR piglet brains using MAP2 (microtubule-associated protein 2) and MBP (myelin basic protein) immunolabelling respectively. Alterations to $GABA_A$ receptor function via changes to receptor subunit expression in the IUGR brain may lead to abnormal neurotransmission. GABA neurotransmission has been shown to impact myelination in the developing brain (Arellano et al., 2016; Zonouzi et al., 2015). The

newborn piglet is an appropriate model of the human neonate comparing favourably in brain development and maturation, lung maturity and cardiovascular function (Dobbing and Sands, 1979; Eiby et al., 2013; Pond et al., 2000). In the IUGR brain, alterations to the normal developmental expression of the $GABA_A$ receptor α_1 and α_3 subunits may impact neuronal cytoskeleton development and myelination. The parietal cortex is part of the central somatosensory system which, together with the hippocampus, are vital for perception, learning and cognitive functions (Volpe, 2008a). These regions are significantly impaired in IUGR animal models (Miller et al., 2014).

2. Experimental Procedures

2.1 Animals and tissue preparation

Large-white piglets (n=65) were obtained from The University of Queensland Gatton Piggery. Approval for this study was granted by The University of Queensland Animal Ethics Committee and was carried out in accordance with National Health and Medical Research Council (NHMRC) guidelines (Australia).

Term (P0 – normally grown (NG) n=10; IUGR n=9) and week-old piglets (P7 – NG n=10; IUGR n=10) were born spontaneously. Caesarean sections were performed on pregnant sows at 100 days of gestation (NG, n=7; IUGR, n=6) and at 104 days of gestation (NG, n=7; IUGR, n=6) (Kalanjati et al., 2011). Both spontaneously born and c-section delivered animals were collected randomly across several litters. Following delivery piglets were resuscitated, weighed and then euthanased via an intracardiac injection of sodium pentobarbital (650 mg/kg; Lethabarb, Virbac, Australia). The brain was immediately removed, weighed, hemisected and coronally sliced. Parietal cortex and hippocampus from the left hemisphere were frozen in 0.32 M sucrose and stored at –80°C while the right hemisphere sections were fixed in 4% paraformaldehyde as previously described (Kalanjati et al., 2011). IUGR piglets were defined by brain to liver weight ratio at birth (BLR) ≥ 1 and by birth bodyweight (< 10^{th} percentile for P0 and P7; < 20^{th} percentile for 100d and 104d) (Bauer et al., 1998; Cox and Marton, 2009; Peleg et al., 2006). BLR is used to define asymmetric growth in the IUGR neonate. This is the most common form of growth restriction (affecting 70-80%) known as 'brain-sparing' where the body is disproportionately smaller than the head.

2.2 Protein preparation and Western blotting

Brain tissue from the parietal cortex and hippocampus were homogenised in 10x volume distilled water, centrifuged at 1400 x *g* for 5 min at 4°C and supernatant collected as previously described (Miller et al., 2016). Protein concentrations were determined by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Victoria, Australia). Protein samples were separated by 10% SDS-PAGE, transferred to PVDF membrane and probed with anti-$GABA_A$ receptor α_1 (1:2000, Millipore, USA) and anti-$GABA_A$ receptor α_3 (1:1000, Millipore, USA) as previously described (Kalanjati et al., 2011; Miller et al., 2016). Following incubation with secondary anti-rabbit IgG-peroxidase antibody (1:30 000, Sigma Aldrich, USA) for 1 hour at RT, proteins were visualised on X-ray film with ECL reagent (GE Healthcare, Australia) and quantified with Image-J software. A pooled protein sample of all samples was used on every gel (5, 10 and 20 μg) as a control for quantification as previously described (Goasdoue et al., 2016; Miller et al., 2016).

2.3 Immunohistochemistry

Brain slices (in triplicate) containing parietal cortex and hippocampus from the right hemisphere (n=3 animals from each time point and group) were embedded in paraffin and serially sectioned. For GABA immunohistochemistry sections were cut at 4μm apart and for MAP2 and MBP immunohistochemistry sections were cut 8μm apart. Sections were affixed to Menzel Superfrost Plus adhesive slides and air-dried overnight at 37°C. Prior to antigen retrieval all sections were dewaxed and rehydrated. For $GABA_A$ receptor α_1 and α_3, and MAP2 immunohistochemistry, antigen retrieval was performed at 105°C for 15–20 min in a decloaking chamber (Borg; Biocare Medical, USA). Monoclonal mouse anti-$GABA_A$ receptor α_1 (1:500), polyclonal rabbit anti-α_3 (1:1500) were applied overnight at RT, mouse anti-MAP2 (1:5000; ExBio, Czech Republic) was incubated overnight at 4°C. The following day sections were incubated with secondary anti-mouse antibody (MACH 3 or anti-rabbit, Biocare Medical, CA) for 10 min or 60 min at RT as previously described (Lingwood, 2008). Labelling was visualised with chromagen 3,3'–diaminobenzidine (DAB, Sigma Aldrich, NSW) and sections counterstained with cresyl violet (0.15%).

For MBP immunohistochemistry sections were placed in an antigen retrieval at 85°C for 10 min. Rat anti-MBP (1:4000; Sigma-Aldrich, USA) was applied and sections incubated for 72 h at 34°C followed by incubation with secondary anti-rat antibody (1:500) for 5 hours at 34°C.

Analysis of immunolabelled sections was performed using a light microscope (Olympus BX41) with a DP70 camera. Image analysis of MAP2 and MBP were performed as previously described (Wixey et al., 2011). Briefly, photomicrographs (600 µm x 600 µm) of cortical grey matter in the parietal cortex and hippocampus for MAP2 and subcortical white matter in the parietal lobe for MBP were taken at 100d, 104d, P0 and P7. Three sequential sections from each piglet were analysed using the commercial software National Institutes of Health Image J as described (Wixey et al., 2011). The areal density is expressed as a percentage of 600 μm^2.

2.4 Statistical analysis

Normality testing of sample distributions was done for each age group. One-way ANOVA with the *post hoc* Tukey analysis was used to determine differences between NG and IUGR animals at each gestational/postnatal age and to assess temporal changes in $GABA_A$ receptor, MAP2 and MBP expression. $p<0.05$ was considered significant (Graph Pad Prism 5.0 software, San Diego, California, USA).

3. Results

Body weight and liver weight were significantly lower for IUGR piglets in comparison to NG for all age groups (Table 1). Brain weight was only significantly lower in the P0 and P7 IUGR groups in comparison to respective NG groups. The mean brain to liver weight ratio (BLR) was significantly higher in the IUGR piglets in comparison to NG for all age groups (Table 1).

3.1 Switch in $GABA_A$ receptor α_1 and α_3 protein expression in IUGR piglet brain

We observed a switch in $GABA_A$ receptor α_1 and α_3 subunit protein expression in IUGR parietal cortex and hippocampus between P0 and P7, similar to the NG pattern previously reported by us (Kalanjati et al., 2011). In the parietal cortex, the α_3 protein showed significantly greater expression during gestation compared to α_1 from 100d to P0 (birth) ($p<0.05$; Figure 1A). The switch from α_3 to α_1 expression in the parietal cortex appeared to occur at around P4-5 in IUGR animals and, by P7 the cortical expression of the α_3 protein was lower than the α_1 protein, however this difference was not significant (Figure 1A). In the IUGR hippocampus, a similar effect was evident at the gestational time-points studied with greater α_3 expression (although not significant) compared to α_1 until P0. Unlike the parietal

cortex the switch from α_3 to α_1 expression in the hippocampus occurred very soon after birth at approximately P1, whereupon α_3 expression significantly declined compared to α_1 by P7 ($p<0.05$; Figure 1B). Representative western blots are shown in Figure 1C.

3.2 Ratio of $GABA_A$ receptor α_1/α_3 protein expression

Analysis of the ratio of $GABA_A$ receptor protein subunit expression levels α_1:α_3 (α_1/α_3) in IUGR parietal cortex compared with normally grown animals was not significantly different in the 100d, 104d and P0 groups. However, at P7 the α_1/α_3 subunit ratio in IUGR parietal cortex was significantly lower when compared with P7 NG cortex ($p<0.05$) (Figure 2A). In hippocampus, the α_1/α_3 subunit ratio in IUGR groups was not significantly different compared with NG animals at any time-point studied (Figure 2B). In contrast to the parietal cortex, the α_1/α_3 subunit ratio in the P7 IUGR hippocampus was not significant although this ratio was increased when compared with P7 NG hippocampus.

3.3 $GABA_A$ receptor α_1, and α_3 immunolabelling

There were no obvious differences in the laminar and cellular distributions of $GABA_A$ receptor α_1 and α_3 subunits in IUGR piglet parietal cortex and hippocampus across any of the age groups studied (Figure 3). The pattern of staining in the IUGR piglet brain was similar to our previous observations in the NG piglet brain (Kalanjati et al., 2011). Immunolabelling of the α_1 and α_3 subunits were shown throughout all cortical layers with the α_3 subunit predominantly observed in layer V-VI. The α_1 subunit was widely distributed in all strata in the hippocampus of IUGR piglets, whereas α_3 subunit expression was limited. At the cellular level, these subunits were observed in the neuropils, cell bodies and processes of pyramidal and non-pyramidal neurons in both parietal cortex and hippocampus. Figure 3 shows a visual increase in α_1 immunolabelling (A-D) and decrease in α_3 immunolabelling (F-I) from 100d to P7 in the IUGR piglet brain.

3.4 MAP2 immunolabelling in the parietal cortex and hippocampus

Impaired MAP2 immunolabelling was observed at all time points in the IUGR parietal cortex and CA1 region of the hippocampus when compared to the same regions in the NG piglet brain (data only shown for P7 – Figure 4). Compared with NG parietal cortex (Figure 4A), the pattern of immmunolabelling in IUGR parietal cortex appeared disrupted showing diminished somatodendrites or 'unhealthy' broken-looking dendrites (Figure 4B). In the hippocampus, there was a distinct loss of somatodendritic labelling by MAP2 in the CA1

region in IUGR animals (Figure 4D) when compared with NG animals (Figure 4C). In all but one age, there were significant reductions in MAP2-positive immunolabelling in the IUGR piglet brain at 104d, P0 and P7 in both the parietal cortex (47.0%, 35.5%, 37.7% respectively; Table 2) and hippocampus (39.6%, 29.8%, 36.0% respectively; Table 2) in comparison to NG piglets.

3.5 MBP immunolabelling in the parietal cortex and hippocampus

MBP immunolabelling was performed in subcortical whiter matter of the parietal lobe of NG (Fig 5A, C, E, G) and IUGR (Fig 5B, D, F, H) piglet brains. In NG brains, MBP immunoreactivity was associated with white matter fibres, typical of the myelination pattern for a developing brain. In IUGR brains (Fig 5 B, D, F, H), immunoreactivity for MBP was decreased and the myelination pattern of the white matter appeared disrupted with marked loss of axonal fibres. The myelinated-axonal fibre pattern in the subcortical white matter of the parietal lobe of NG piglets was developmentally regulated from 100d to P7, where expression increased gradually from 100d to P7 (Fig 5A, C, E, G; Table 2). However, significant reductions in MBP-positive immunolabelling of myelinated-axonal fibres in the subcortical white matter of the parietal lobe were evident in the IUGR piglet brain at 104d, P0 and P7 (51.1%, 22.7%, 32.8% respectively; Table 2) in comparison to NG piglets.

4. Discussion

A switch in the dominant expression between $GABA_A$ receptor α_3 and α_1 subunits occurs around birth in multiple mammalian species including the piglet brain (Brooks-Kayal and Pritchett, 1993; Chen et al., 2001; Kalanjati et al., 2011; Laurie et al., 1992; Liu and Wong-Riley, 2004; McKernan et al., 1991; Takayama and Inoue, 2004). In the current study, we report altered $GABA_A$ receptor α subunit expression in IUGR animals at birth and at 1 week postnatal age as well as changes to neuronal cytoskeletal structure and myelination across several gestational time-points.

Unlike our findings in the parietal cortex of NG animals, which showed a significant upregulation of α_1 expression by P7, in IUGR animals, α_1 expression did not upregulate significantly between P0 and P7 congruent with the normal developmental maturation of the $GABA_A$ receptor neurotransmission system (Kalanjati et al., 2011). The reverse was true however for the hippocampus where we found a significant acceleration of α_1 expression in

IUGR animals from P0 to P7 which was not evident in NG animals. $GABA_A$ receptor α_3 subunit expression in the P0 and P7 IUGR parietal cortex did not a downregulation, which is present in NG animals at this stage of brain development (Kalanjati et al., 2011); hippocampal α_3 expression did not differ between IUGR and NG animals. While in the parietal cortex there appeared to be a delay in upregulation of the "mature" subunit α_1 and/or prolonged expression of "embryonic" subunit α_3 in IUGR animals, in the hippocampus the switch in expression between α_1 and α_3 appeared to occur faster in IUGR animals. Neither subunit appeared to differ in their distribution in the parietal cortex or hippocampus of IUGR piglets at any of the age groups studied when compared to our previous findings in NG animals (Kalanjati et al., 2011).

Such alterations to $GABA_A$ receptor α subunit expression may have important impacts on the developing brain. A switch in the dominance of the α subunit expressed between α_3 and α_1 is in line with the switch in GABA neurotransmission from excitation to inhibition during brain maturation. Furthermore, alterations in $GABA_A$ receptor subunit protein expression modify the receptor subtype, which in turn results in changes to $GABA_A$ receptor kinetics, pharmacological properties and neurotrophic function (Belhage et al., 1988; D'Hulst et al., 2009; Doris and Arnold, 2009; Lavoie et al., 1997; Serafini et al., 1998; Verdoorn et al., 1990) . The altered $GABA_A$ subunit expression pattern in P7 IUGR piglet parietal cortex found in our study may not only affect GABA neurotransmission but may result in alterations to normal cortical development increasing the risk of neuropathological conditions. In humans, temporal and regional expression of specific $GABA_A$ receptor subunits correlates with the development of GABAergic interneurons and their thalamocortical projections (Houser et al., 1988; Tiu et al., 2002; Volpe, 2008a; Zecevic and Milosevic, 1997; Zezula et al., 1988).

In the parietal cortex and CA1 hippocampus of IUGR animals, we observed obvious impairments to neuronal somatodendrites. A dramatic loss of neuronal somatodendrites labelled by MAP2 were observed across several gestational time points (104d, P0 and P7) suggesting that chronic growth restriction disrupts neuronal cytoskeletal architecture in IUGR. In acute HI, MAP2 is a sensitive marker for cerebral HI damage. Previous work in our laboratory in the neonatal HI piglet model showed severe impairments to MAP2 labelling with marked loss of dendrites and increased pyknotic neuronal nuclei in almost all layers of parietal cortex and hippocampus (Lee A, 2010; Lingwood et al., 2008). In gerbil cortex and

CA1 hippocampus, loss of neuropils, cell bodies and dendrites of neurons are seen after ischemia for 30 min (Kitagawa et al., 1989), while reduced MAP2 labelling has been found after acute HI in rat cortex (Blomgren et al., 1995). A more subtle and longer period of hypoxia and nutrient depletion such as is evident in IUGR appears to result in more subtle disturbances to neuronal somatodendrites that may impact normal cortical development. However in our current study we did not examine beyond postnatal day 7. Examining a later time point as well as performing neurobehavioral assessments would reveal whether this neuronal injury has long lasting effects and whether these disturbances result in long term neurodevelopmental issues.

Additionally, in mice lacking the $GABA_A$ receptor α_1 subunit, neuronal dendritic filopodia have been reported to be increased although the mature mushroom-shaped spines of these dendrites were found to be significantly decreased (Heinen et al., 2003). By binding to $GABA_A$ receptors, GABA may stimulate the neuritic outgrowth in rat hippocampus and modulate the plasticity of superior cervical ganglionic cell dendrites in adult rat (Barbin, 1993; Wolff, 1978). Thus hypoxia and altered $GABA_A$ receptor expression may independently and/or together impair the neuronal somatodendritic expressions observed in IUGR piglet parietal cortex and hippocampus.

In our current study we also observed considerable reduction in MBP immunoreactive fibres in the subcortical white matter of the parietal lobe of IUGR piglets from 104d, suggesting myelination is also susceptible to injury from the effects of IUGR. 104d in the piglet is similar to a human preterm infant between 26-28 weeks gestation (Eiby et al., 2013). At this time point brain growth and myelination are occurring at a rapid rate (Kinney et al., 1994). In fact, myelination in the fetal piglet brain has been shown to increase at a rapid rate between 100d and 110d (Pond et al., 2000). In preterm human infants an arrested stage of active myelination between 23-36 weeks of gestation has been found to correlate with the incidence of periventricular leukomalacia (PVL), with death of pre-oligodendrocytes (that are highly susceptible to hypoxia) postulated as the underlying mechanism (Back et al., 2002; Back and Volpe, 1997; Hagberg et al., 2002; Volpe, 2008b). Selective death of pre-oligodendrocytes was previously considered the main mechanism underlying deficits in myelination, however recent studies in both premature human infants (Billiards et al., 2008; Verney et al., 2012) and in animal models of IUGR (Reid et al., 2012; Tolcos et al., 2011) suggest oligodendrocytes are arrested at the premyelinating stage and fail to fully mature into

myelinating oligodendrocytes. Furthermore after acute injury such as HI in rats, acute loss of oligodendrocytes is followed by proliferation of the oligodendrocyte progenitor cells that cannot differentiate further into mature oligodendrocytes (Cheng et al., 2015).

In accordance with our demonstrated disruption and loss of neurons and white matter, clinical imaging studies demonstrate alterations in brain structure in IUGR infants including altered white and grey matter volumes, decreased levels of brain connectivity, decreased cortical thickness and delayed cortical development (Esteban et al., 2010; Padilla et al., 2015; Tolsa et al., 2004). In IUGR infants born preterm (26-34 wks GA), magnetic resonance imaging (MRI) at 12 months corrected age shows areas of decreased grey matter and white matter volumes with decreases in the cerebellum and hippocampus (Padilla et al., 2011). These abnormal patterns may reflect abnormal neuronal activity and myelination and therefore abnormal functionality in the IUGR brain. These abnormal neuronal and/or white matter patterns have also been demonstrated in small animal models of IUGR (Wixey et al., 2016) with loss of oligodendrocytes, decreased proliferation and differentiation reported (Mazur et al., 2010; Olivier et al., 2005; Pham et al., 2015; Tolcos et al., 2011). Yet some studies show postnatal restoration of white matter (Olivier et al., 2007; Reid et al., 2012; Tolcos et al., 2011). However even though myelination abnormalities were resolved, functional deficits were still evident in 8 week IUGR rats (Reid et al., 2012). Thus disruption to myelination prior to birth may result in ongoing neuropathological effects in the neonate.

Our understanding of the signaling mechanisms mediated by GABA in the role of myelination in the IUGR brain is poor. We can gather information from recent neonatal studies where $GABA_A$ receptors have been shown to play a role in oligodendrocyte differentiation and proliferation in the neonatal brain (Zonouzi et al., 2015). GABAergic signaling regulates oligodendrocyte progenitor cell differentiation and proliferation in the preterm neonatal brain and may be a key factor in diffuse white matter injury in these neonates (Zonouzi et al., 2015). Therefore it seems plausible GABA could play a similar role in the IUGR brain. However the white matter disruption in the IUGR piglet brain preceded the disruption to $GABA_A$ α subunit expression which was not observed until after birth at postnatal day 7. Functional GABAergic studies and $GABA_A$ and oligodendrocytes co-localisation studies would be beneficial to determine the mechanisms behind this disruption. Nonetheless, with altered $GABA_A$ receptor actions and disruption to myelination during a critical window of development this may impair functional outcomes in IUGR neonates.

The parietal cortex contains the somatosensory cortex and the hippocampus is important for memory function and forms part of the limbic system (Volpe, 2008a). Significant alterations to $GABA_A$ receptor α_1 and α_3 protein expression levels were observed in the IUGR piglet. Additionally, we found that neuronal somatodendrites and myelinated-axonal fibres are impaired in IUGR brain at certain gestational time-points, with the parietal cortex and CA1 hippocampus showing a higher vulnerability. These cellular impairments may disrupt proper synaptogenesis and neurotransmission crucial to normal brain function. Elucidating the mechanisms behind grey and white matter injury in the IUGR infant may help our understanding of the cognitive impairment in IUGR and is essential to identifying targets for treatments to improve long-term outcomes for IUGR infants.

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Figure Legends

Figure 1. $GABA_A$ receptor subunit α_1 and α_3 protein expression in IUGR cerebrum between 100d and P7 in the parietal cortex (A) and hippocampus (B) in IUGR piglets. A switch of predominant expression between these two subunits occurs around birth (P0) in the NG piglet brain (Kalanjati et al., 2011), however this switch was not significant at P7 in the IUGR parietal cortex (A). In the hippocampus the switch after birth resulted in a significant increase in α_1 and decrease in α_3 expression (B; $p<0.05$). (C) Representative western blots of α_1 (lane 1: P-10 NG hippocampus; lane 2: P-10 IUGR hippocampus; lane 3: P7 NG hippocampus; lanes 4-6 are standards: 20, 10, 5 μg) and α_3 (Lane 1: P7 NG hippocampus; lane 2: P0 NG hippocampus; lane 3: P7 IUGR parietal cortex; lanes 4-6 are standards: 20, 10, 5 μg). Values are the mean ± S.E.M. $^*p < 0.05$ α_1 versus α_3.

Figure 2. The α_1/α_3 subunit ratio in 100d, 104d, P0 and P7 NG and IUGR parietal cortex (A) and hippocampus (B) in NG and IUGR piglets. The α_1/α_3 ratio was significantly lower in IUGR cortex compared to NG cortex in the P7 group ($p<0.05$). There were no significant differences between NG and IUGR groups at all ages. Values are the mean ± S.E.M. $^*p <$ 0.05, IUGR versus NG.

Figure 3. $GABA_A$ receptor α_1 and α_3 subunit distribution in IUGR piglet brains at 100d (A&F), 104d (B&G), P0 (C&H) and P7 (D&I) respectively. Greater grey matter α_1 immunolabelling was present in the cortex (arrow, D) compared to the white matter. At a cellular level α_1 immunolabelling was observed throughout cortical layers (inset, E) with labelling seen in neuropils, cell bodies and membranes of pyramidal and non-pyramidal neurons (arrows, E). In the hippocampus, α_1 immunolabelling was shown in CA1, CA3 and the dentate gyrus (* in B). Greater grey matter α_3 immunolabelling was shown in the cortex (arrow, F) compared to white matter. α_3 immunolabelling was predominant in the deeper layers (V-VI) compared with the more superficial layers (arrowhead inset, J) from neuropils,

neuronal membranes and processes labelling from layers V-VI. A streaky fibre labelling pattern was also seen from the neuronal processes in layers V-VI to the more superficial layers (arrow, J). In the hippocampus, α_3 immunolabelling was sparse (* in I). Scale bar: 0.5 cm (A-D, F-I); 200 μm (E&J)

Figure 4. MAP2 immunolabelling in P7 piglet parietal cortex and CA1 hippocampus. In NG piglets, the immunolabelling was observed on the somatodendrites of neuronal cells (arrow, A-D). In IUGR piglets, the MAP2 immunolabelling pattern in the cortical layers was impaired, demonstrated either as a diminished somatodendritic pattern of the neurons or as an unhealthy-broken looking pattern of the dendrites (arrow, B). Fewer somatodendrites of neuronal cells were seen in IUGR CA1 layers (arrow, D) when compared to NG CA1 (E&G). Scale 100 μm.

Figure 5. Representative photomicrographs of MBP-immunolabelling in piglet subcortical white matter of the parietal lobe at 100d NG (A), 100d IUGR (B), 104d NG (C), 104d IUGR (D), P0 NG (E), P0 IUGR (F), P7 NG (G), and P7 IUGR (H). In NG brains (A-D), MBP immunoreactivity was associated with white matter fibres, typical of the myelination pattern for a developing brain. In IUGR brains (E-F), immunoreactivity for MBP was decreased and the myelination pattern of the white matter appeared disrupted with marked loss of axonal fibres. Scale bar 200 μm.

Figure 1

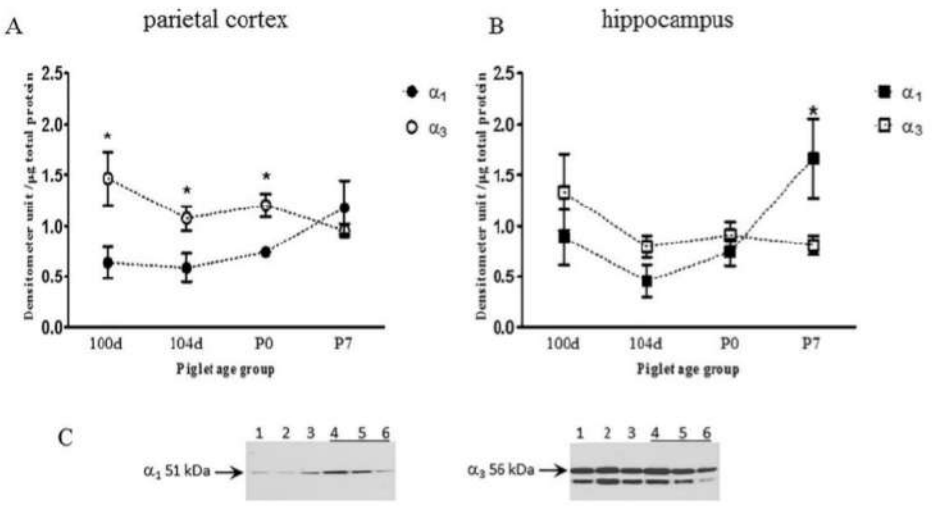

Figure 2

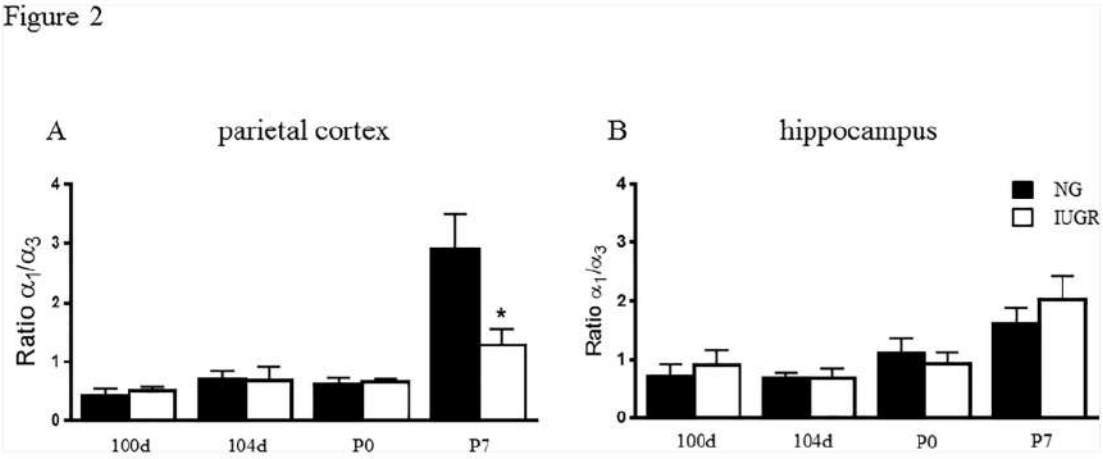

Figure 3

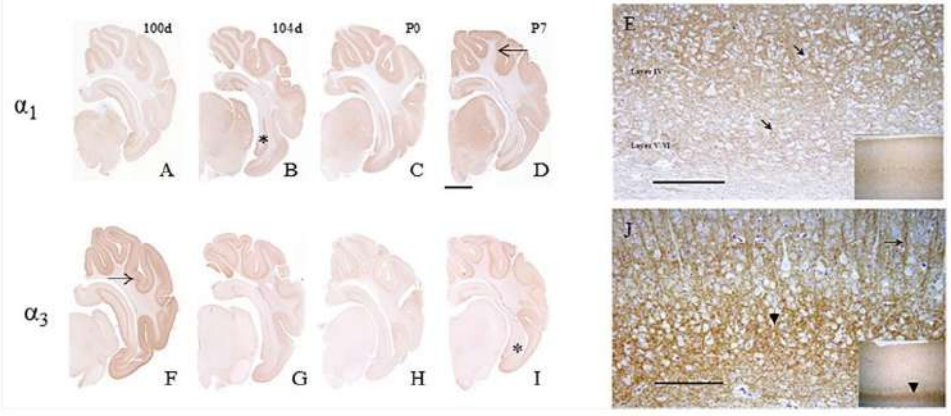

Figure 4

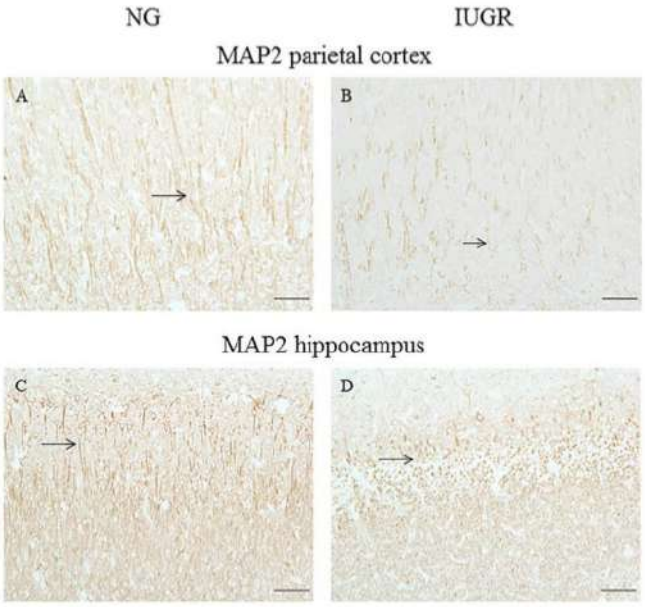

Figure 5

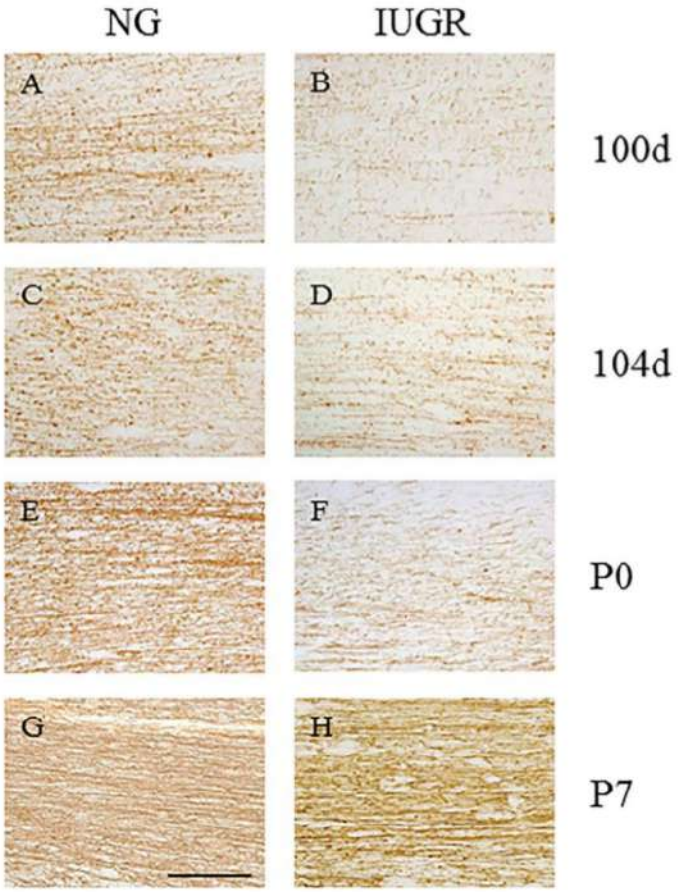

Table 1. Piglet bodyweight, brain weight and liver weight. IUGR piglets in all groups had significantly lower mean bodyweight and liver weight when compared to NG piglets from each respective age group ($p<0.05$). When compared to NG groups, significantly lower mean brain weight was observed in only the P0 and P7 groups ($p<0.05$); but not in the preterm groups. However IUGR piglets in all age groups demonstrated significantly higher brain to liver weight ratio in comparison to respective NG groups ($p<0.05$). Values are the mean ± S.E.M. $^{*}p < 0.05$ lower value in IUGR versus NG. $^{\dagger}p < 0.05$ higher value in IUGR versus NG.

Piglet groups	**Bodyweight in grams (mean ± SD)**	**Brain weight in grams (mean ± SD)**	**Liver weight in grams (mean ± SD)**	**Brain:liver ratio (mean ± SD)**
100d				
NG (n=7)	981.4 ± 171.8	24.6 ± 3.7	23.6 ± 5.3	1.06 ± 0.15
IUGR (n=6)	619.2 ± 65.9*	23.8 ± 2.6	15.8 ± 3.9*	1.55 ± 0.29†
104d				
NG (n=7)	1281.3 ± 136.1	26.4 ± 1.8	28.6 ± 4.6	0.95 ± 0.17
IUGR (n=6)	815.0 ± 127*	25.0 ± 1.4	18.7 ± 2.8*	1.37 ± 0.21†
P0				
NG (n=10)	1406.9 ± 221.2	32.3 ± 1.9	51.6 ± 8.6	0.64 ± 0.09
IUGR (n=9)	769.2 ± 117.8*	29.4 ± 3.2*	24.5 ± 7.7*	1.34 ± 0.31†
P7				
NG (n=10)	2710 ± 303.5	39.4 ± 2.7	108.1 ± 24.2	0.38 ± 0.23
IUGR (n=10)	1374.1 ± 451.5*	34.1 ± 2.5*	48.4 ± 26.7*	1.30 ± 0.46†

Table 2. Area density measurements of MAP2 and MBP immunolabelling in parietal cortex and hippocampus. Data are expressed as percentages of 600μm^2 area. *p<0.05, **p<0.01, ***p<0.001 IUGR versus NG

Gestational Age	**MAP2 parietal cortex**		**MAP2 hippocampus**		**MBP parietal cortex**	
	NG n=3	**IUGR n=3**	**NG n=3**	**IUGR n=3**	**NG n=3**	**IUGR n=3**
100d	16.62±1.26	11.16±1.99	16.01±2.77	10.35±1.45	18.41±1.98	12.5±1.58
104d	17.14±0.57	9.09±0.54***	16.08±1.09	9.719±1.07*	19.64±1.84	9.61±1.45**
P0	17.35±0.75	11.19±1.25*	14.9±0.89	10.46±0.52**	23.45±1.77	18.13±0.58*
P7	20.42±1.56	12.72±1.40*	21.18±1.18	13.55±2.25*	29.75±2.41	19.99±0.93*

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GABAa Receptor expression and white matter disruption in intrauterine growth restricted piglets

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