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

Viskasari P. Kalanjati[a,b], Julie A. Wixey[a], Stephanie M. Miller[a], Paul B. Colditz[a], Stella T. Bjorkman[a,*]

[a] The University of Queensland, UQ Centre for Clinical Research, Perinatal Research Centre, Royal Brisbane and Women's Hospital, Brisbane, Queensland, 4029, Australia
[b] Faculty of Medicine, Airlangga University, Surabaya, Indonesia

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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 = 115 d – days gestational age), 100 d, 104 d, 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 104 d 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.

1. Introduction

Intrauterine growth restriction (IUGR) is one of the most common causes of perinatal mortality and morbidity (Allen and Gillespie, 2001; Gillespie and Allen, 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 and Gillespie, 2001; De Bie et al., 2010; Gillespie and Allen, 2002). Hypoxemia and impaired nutrient supply during pregnancy are major contributors to brain pathophysiology in IUGR neonates (Cox and Marton, 2009; Economides et al., 1989; Nicolaides and Soothill, 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

* Corresponding author.
E-mail address: t.bjorkman@uq.edu.au (S.T. Bjorkman).

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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–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 (~30 wk human GA), 104 days gestational age (>32 wk 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 (<10th percentile for P0 and P7; <20th percentile for 100 d and 104 d) (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 10× volume distilled water, centrifuged at 1400*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 h 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 4 μm apart and for MAP2 and MBP immunohistochemistry sections were cut