Brain Structure and Function (2018) 223:1025–1033
https://doi.org/10.1007/s00429-017-1597-6

SHORT COMMUNICATION

Identification and expression of a unique neonatal variant of the $GABA_A$ receptor α_3 subunit

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Received: 29 August 2016 / Accepted: 20 December 2017 / Published online: 27 December 2017

Abstract

The $GABA_A$ receptor provides the majority of inhibitory neurotransmission in the adult central nervous system but in immature brain is responsible for much of the excitatory drive, a requirement for normal brain development. It is well established that $GABA_A$ receptor subunit expression changes across the course of brain development. In the present study, we have identified a splice variant of the $GABA_A$ receptor α_3 subunit which appears unique to the developing brain, referred to here as the $GABA_A$ receptor α_3 subunit neonatal variant ($GABA_A$ receptor α_{3N}). RT-PCR and sequence analysis revealed splicing of exon 8 of the α_3 subunit. Western blot analysis showed expression of $GABA_A$ receptor α_{3N} in the cortex of several neonatal species and significantly reduced expression of this splice variant in the corresponding adult brains. Expression was evident in multiple brain regions and decreased across development in the pig. Fractionation revealed differential cellular localisation in the parietal cortex, hippocampus and thalamus of the full-length $GABA_A$ receptor α_3 and $GABA_A$ receptor α_{3N}. Immunoprecipitation showed direct interaction with the $GABA_A$ receptor subunits α_1 and γ_2 but not with gephyrin.

Keywords $GABA_A$ receptor · Development · Neonatal

Abbreviations

mRNA	Messenger RNA
cDNA	Complementary DNA
$GABA_A$	γ-Aminobutyric acid subtype A
TM	Transmembrane domain

Introduction

The excitatory action of the γ-aminobutyric acid subtype A ($GABA_A$) receptor in neonatal brain plays an important role in influencing the development of the CNS, regulating proliferation of neural and glial progenitors and subsequent migration (Haydar et al. 2000). This early developmental role of the GABA system is reflected in the expression of receptor subunits which differ significantly from the adult brain (Laurie et al. 1992; Pirker et al. 2000).

The $GABA_A$ receptor is an ionotropic chloride channel, formed by 5 subunit proteins of which 16 subunit isoforms have been identified in mammals (α_1–α_6, β_1–β_3, γ_1–γ_3, δ, ε, θ, and π); several splice variants have also been reported (Olsen and Sieghart 2009). In the adult brain, the $GABA_A$ receptor α_3 subunit protein migrates at a mass of approximately 56 kDa. Using a commercially available $GABA_A$ receptor α_3-specific antibody, our laboratory routinely identifies a second highly specific smaller protein in the neonatal pig brain that migrates at approximately 47 kDa (Kalanjati et al. 2011; Miller et al. 2016). The aim of this project was to identify this smaller protein. We hypothesized that this band is a splice variant of the $GABA_A$ receptor α_3 subunit.

Materials and methods

Compliance with ethical standards: tissue

All applicable international, national, and institutional guidelines for the care and use of animals were followed. Animal experiments were undertaken with ethical approvals from the University of Queensland and in accordance

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with National Health and Medical Research Council guidelines (Australia). Total protein samples from human brain were a gift from Assoc. Prof Peter Dodd (University of Queensland, Australia). Total protein samples from rat brain were a gift from Dr Julie Wixey and Prof Karen Moritz (University of Queensland, Australia).

RT-PCR analysis

Total RNA was extracted from 91 days gestational age (GA), 100 days GA (full-term = 115 days GA), postnatal day 4 (P4) and adult pig cortex using the RNeasy Midi Purification Kit (Qiagen, Chadstone, VIC, Australia) according to the manufacturer's instructions. Following extraction, total RNA was denatured at 65 °C for 5 min and cooled on ice. cDNA was synthesized (50 °C for 1 h, 70 °C for 15 min) using an oligo dT primer and SuperScript III reverse transcriptase (Invitrogen Life Technologies, Thornton, NSW, Australia). RNase H was added to remove complementary RNA and the cDNA incubated at 37 °C for 20 min.

Primers were designed against the full coding region human of the $GABA_A$ receptor α_3 subunit (Table 1). PCR amplification was performed in a final reaction volume of 50 µL as follows: 2.5U BioTaq DNA Polymerase, 1× NH_4 reaction buffer, 0.2 µM forward and reverse $GABA_A$ receptor α_3 subunit primers, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and RNase/DNase-free dH_2O; a control sample without cDNA was also included in every experiment. Samples were run on a thermal cycler under the following conditions: denaturation 95 °C for 2 min, followed by 35 amplification cycles (95 °C for 30 s, 60 °C for 30 s, 72 °C for 1.5 min, and 72 °C for 5 min).

Gel extraction and sequencing

PCR products were resolved on a 0.8% agarose gel at 70 V for 2 h. Gels were stained with 0.5 mg/ml ethidium bromide (Sigma-Aldrich, Castle Hill, NSW, Australia) and PCR products visualised using the BioRad Gel Doc system. The PCR product of interest was excised from the gel and purified using the QiaQuick Gel Extraction Kit according to the manufacturer's instruction (Qiagen). Overlapping primers were designed to cover approximately 400 bp sections of the $GABA_A$ receptor α_3 subunit human sequence to ensure full coverage of the coding region (Table 1). The product was sequenced using a 3730 Genetic Analyser (Applied Biosystems, distributed by Thermo Fisher Scientific, Brendale, QLD, Australia) and the sequence analysed using Mutation Surveyor (Softgenetics, State College, PA, USA) and Seqscape V26 (Applied Biosystems).

Total protein preparations and cellular fractionation

For total protein preparations, brain tissue was homogenised in 10× volume ddH_2O, centrifuged at 1400×*g* for 5 min at 4 °C and the supernatant collected. Protein concentrations for all samples were determined by bicinchoninic acid (BCA) assay according to manufacturer's instruction (Pierce BCA Protein Assay, distributed by Thermo Fisher Scientific).

For cellular fractionation pig parietal cortex, hippocampus and thalamus samples were homogenised in 5× volume of homogenising buffer (0.32M sucrose, 2 mM EDTA, 10 mM HEPES, dH_2O) with protease inhibitors (complete EDTA-free protease inhibitor cocktail, Sigma-Aldrich). Samples were initially centrifuged at 6300×*g* for 10 min at 4 °C. The resulting supernatant (S1) was spun at 107,000×*g* for 30 min to obtain the cytosolic fraction (S2) and the

Table 1 Primer pairs designed for amplification and sequencing of the human $GABA_A$ receptor α_3 sequence (Genbank Ref. #:NM_000808.3)

Primer	Sequence (5′–3′)	Start (bp)	Stop (bp)	Expected size (bp)
Forward 1	GCTCGGTCTCTCCAAGTTTGT*	210	230	350
Reverse 1	CTGACACAGGGCCAAAACTGGT	538	559	
F2	GCAGGACATTGGCGGGCTGT	366	385	414
R2	AGGAGGGTTCCGTTGTCCACCA	758	779	
F3	GACCACGCCCAACAAGCTGC	732	751	328
R3	TCGCTTGAGATGGAAGTGGGTTG	1037	1059	
F4	TGGGCCATGTTGTTGGGACAGA	977	998	400
R4	TCCAGGGCCTCTGGCACCTT	1357	1376	
F5	TTTCACCAAGCGGAGTT	1323	1339	392
R5	ACCACTATCTACTGTTTGCGG*	1707	1727	

Forward (F1) and reverse (R5) primers designated with * were used for initial PCR amplification of the full-length α_3 CDS

membrane fraction (P3). The pellet from the initial centrifugation (P1) was resuspended in homogenisation buffer and spun at 4000×*g* for 5 min at 4 °C to obtain the nuclear fraction (P2). Protein concentration estimates were determined by BCA assay as described above.

Western blotting

Total protein samples (10 µg) and fractionated samples (membrane and nuclear fraction—5 µg; cytosolic fraction—20 µg) were separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) at 200 V for 50 min and then transferred to polyvinylidiene fluoride membrane (0.45 µm Immobilon PVDF, Merck Millipore, Bayswater, VIC, Australia) at 100 V for 1 h. Prior to application of primary antibody, membranes were blocked 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) for 1 h with agitation. Membranes were probed overnight at 4 °C on an orbital shaker with rabbit polyclonal anti-$GABA_A$ receptor α_3 (1:15,000 for total protein (Fig. 2), 1:30,000 for fractionated samples (Fig. 4); Merck Millipore #AB5594) which is directed toward the N-terminus of the human sequence (Fritschy and Mohler 1995) (see Fig. 1c). Previous concentrations for this antibody have been reported at 1:10,000 in both western blotting and immunohistochemistry (Chen et al. 2001; Fritschy and Mohler 1995). Membranes were washed 3 × 10 min in TBST, followed by incubation with secondary anti-rabbit IgG-peroxidase antibody (1:50,000; #A0545, Sigma-Aldrich) for 1 h at room temperature (25 °C). The membranes were washed again as before and proteins visualised by enhanced chemiluminescence on X-ray film following incubation with Luminata Forte (Merck Millipore). A second primary antibody directed against the cytoplasmic loop (Fig. 1c) of $GABA_A$ receptor α_3 (Santa Cruz Biotechnology #sc-31410) was also tested. Total protein expression was quantitated by densitometry analysis with the Image-J software (National Institutes of Health, MD, USA) and the relative level of protein expression determined from a standard curve generated from pooled samples run on each blot (Goasdoue et al. 2016; Miller et al. 2017). $GABA_A$ α_{3N} variant was then expressed as a percentage of full-length $GABA_A$ receptor α_3 expression at each individual developmental age.

Immunoprecipitation

Fresh cortex (newborn term pig—P0) was rinsed three times in ice-cold 0.1M phosphate-buffered saline then homogenised in immunoprecipitation lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40) with protease inhibitors (cOmplete EDTA-free Protease inhibitor cocktail, Sigma-Aldrich). Homogenates were mixed for 2 h at 4 °C, then centrifuged at 17,000×*g* for 25 min at 4 °C and the supernatant (lysate) collected. Brain lysates were precleared with Protein A-agarose beads (#16–156, Merck Millipore) at 4 °C for 1 h, then separately incubated with primary antibodies overnight at 4 °C with agitation; anti-$GABA_A$ receptor α_3 (AB5594),

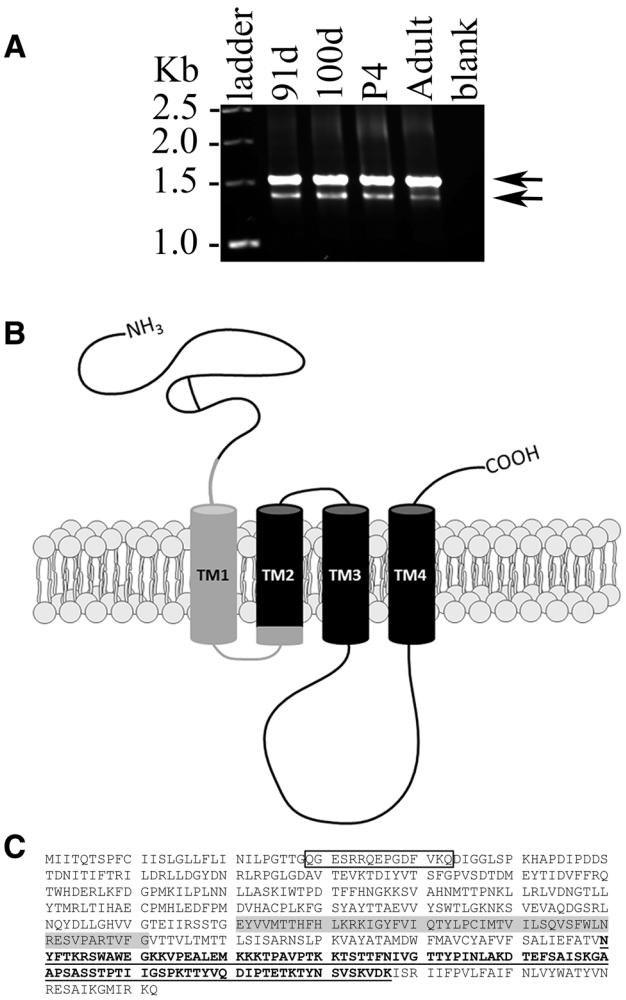


Fig. 1 Identification of the $GABA_A$ receptor α_3 subunit splice variant. **a** RNA extracted from pig parietal cortex at fetal (91 and 100 days GA) and postnatal (P4 and adult) stages of development were used for RT-PCR analysis with α_3 specific primers F1 and R5 (see Table 1). Analysis of PCR products on a 1% agarose gel showed the expected 1530 bp fragment (upper band) and a smaller ~ 1300 bp fragment (lower band) corresponding to wild-type α_3 and an α_3 splice variant, respectively, at all developmental ages. A PCR negative control (blank) is also shown. **b** Model of the topology of the $GABA_A$ receptor α_3 subunit protein. Splicing of exon 8 results in loss of amino acids 260–310 which encompasses all of TM1 and partial loss of TM2 (grey shading). **c** Protein sequence of pig $GABA_A$ receptor α_3 subunit. Highlighted grey sequence corresponds to amino acids that code for exon 8. The boxed region corresponds to the sequence against which the $GABA_A$ receptor α_3 Merck-Millipore antibody has been raised (#AB5594). The underlined region corresponds to the cytoplasmic loop between TM3 and TM4 and the region against which the Santa Cruz $GABA_A$ receptor α_3 antibody is directed (#sc-31410)

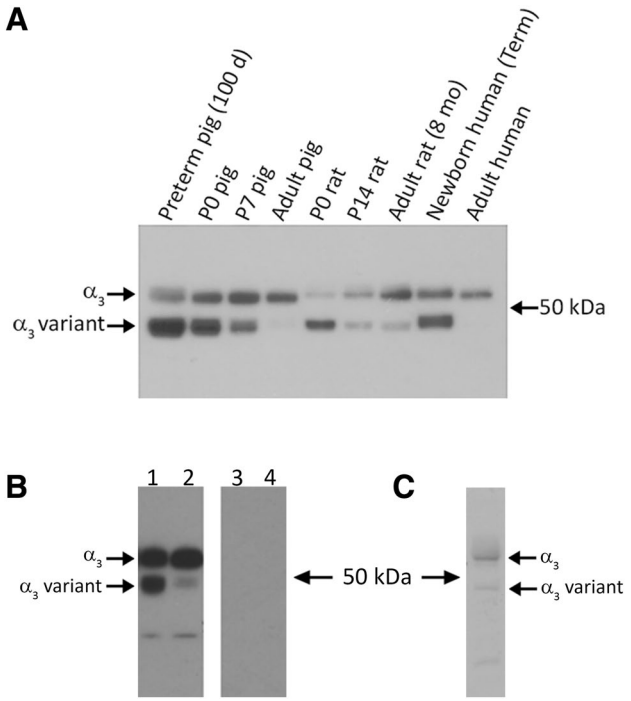


Fig. 2 Representative western blots. **a** Expression of $GABA_A$ receptor α_3 total protein (~56 kDa) and the α_{3N} variant (~47 kDa) in neonatal and adult cortex of the pig, rat and human. **b** Antibody specificity was confirmed with pre-absorption using the Merck-Millipore (#AB5594) control antigen; lane 1; P7 pig; lane 2: adult pig; lane 3: pre-absorption P7 pig; lane 4: pre-absorption adult pig. **c** A separate antibody directed toward the cytoplasmic loop of the $GABA_A$ receptor α_3 protein (Santa Cruz Biotechnology #sc-31410) also confirmed the presence of the full-length and variant protein

mouse monoclonal anti-$GABA_A$ receptor α_1 (#MAB339, Merck Millipore), rabbit polyclonal anti-$GABA_A$ receptor γ_2 (#AB5954, Merck Millipore), mouse monoclonal anti-Gephyrin (#147-021, Synaptic Systems, Goettingen, Germany) and Rabbit IgG (#I5006, Sigma-Aldrich). Protein A-agarose beads (#16–156) were added to each lysate and the immunocomplex captured with gentle rotation at 4 °C for 2 h. The protein A-agarose beads were then washed five times in IP lysis buffer, spun and SDS sample buffer added to the pellet and heated to 95 °C for 5 min. Western blotting was performed as described above and membranes probed with anti-$GABA_A$ receptor α_3 (1:15,000 #AB5594), rabbit polyclonal anti-$GABA_A$ receptor α_1 (1:3,000 #AB5609, Merck Millipore), or rabbit polyclonal anti-$GABA_A$ receptor γ_2 (1:2000 #AB5559, Merck Millipore) primary antibodies.

Results

Total RNA from pig cortex was extracted, reverse transcribed and the cDNA amplified with α_3 primers specific to the full-length human α_3 mRNA-coding sequence. We amplified the expected 1530 bp product for the full-length $GABA_A$ receptor α_3 subunit (GenBank accession number KY595782) as well as a smaller product of approximately 1300–1400 bp at all developmental ages (Fig. 1a). Sequence analysis of this smaller product showed that it was a splice variant (1479 bp) of the $GABA_A$ receptor α_3 subunit. Alignment with the native human and pig $GABA_A$ receptor α_3 subunit revealed that the α_3 variant lacked exon 8, herein named $GABA_A$ receptor α_3 subunit neonatal variant ($GABA_A$ receptor α_{3N}) (GenBank accession number KX671124). Exon 8 of the $GABA_A$ receptor α_3 subunit is comprised of 153 bp; deletion of exon 8 does not alter the reading frame.

The full-length $GABA_A$ receptor α_3 subunit codes for 492 amino acids in both pig and human with a predicted molecular weight of 55 kDa. The amino acid sequence of the $GABA_A$ receptor α_{3N} variant is virtually identical except for the lack of 51 amino acids resulting in a protein with a predicted molecular weight of 49 kDa. Exon 8 encodes for the probable transmembrane domain 1 (TM1) and part of TM2 (Fig. 1b, c).

Western blot analysis of total protein samples from cortex revealed that the $GABA_A$ receptor α_{3N} variant is also present in both developing human and rat brain with near undetectable levels in the adult brains of pig, rat and human (Fig. 2a). Antibody specificity was confirmed with pre-absorption using the Merck-Millipore (#AB5594) control antigen (Fig. 2b). A separate antibody directed toward the cytoplasmic loop of the $GABA_A$ receptor α_3 protein (Santa Cruz Biotechnology #sc-31410) was also used to confirm the presence of the variant protein and to eliminate non-specific antibody reactivity due to differing epitopes (Fig. 2c). $GABA_A$ receptor α_{3N} shows widespread distribution across the brain. Analysis of the α_{3N} variant from total protein homogenates shows differential expression across several brain regions and gestational ages (Fig. 3) similar to that reported for the full-length $GABA_A$ receptor α_3 protein; full-length $GABA_A$ receptor α_3 expression is greater during fetal development and declines with age, particularly in cortical regions (Laurie et al. 1992; Miller et al. 2017).

$GABA_A$ α_{3N} variant was expressed as a percentage of full-length $GABA_A$ receptor α_3 expression at each individual developmental age (Fig. 3). The $GABA_A$ α_{3N} variant was highly expressed during fetal brain development in cortical regions but expression gradually declined with advancing gestation and by adulthood was substantially lower (Fig. 3). In the hippocampus, α_{3N} expression varied somewhat across development, but maintained a relatively modest level of expression through to adulthood. In basal ganglia, thalamus and cerebellum levels remained relatively low but ubiquitous across all gestational ages into adulthood, while full-length α_3 expression decreased with age (Miller et al. 2017).

Further western blot analysis of fractionated brain tissue samples from parietal cortex, hippocampus and

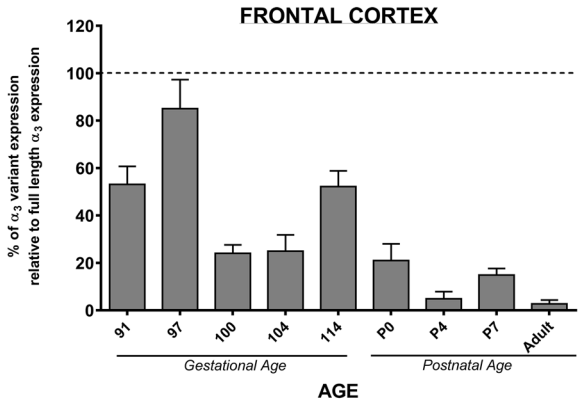
thalamus showed that the $GABA_A$ receptor α_3 protein and the $GABA_A$ receptor α_{3N} variant were not only differentially expressed across the subcellular fractions, but also across fetal and postnatal ages (Fig. 4). Both the full-length $GABA_A$ receptor α_3 protein and α_{3N} variant were found to localise predominantly to the membrane and nuclear fractions across all ages, although α_{3N} variant expression was higher in developing fetal brain (91 GA and 100 days GA) compared with postnatal ages (P4 and adult). Cytosolic expression of both the α_3 protein and the α_{3N} variant was much lower compared with the membrane and nuclear fractions; however, there was a distinct switch in cytosolic expression between the α_3 and α_{3N} variants. In the hippocampus and thalamus, full-length cytosolic α_3 was expressed almost exclusively in fetal brain, while in the postnatal brain, the α_{3N} variant was exclusively expressed in cytosolic fractions. In the parietal cortex, there was overlap of α_3 and α_{3N} expressions closer to birth but by adulthood in all brain regions studied, only the α_{3N} variant was expressed in the cytosol. Differences in methodology should also be noted with regard to the results presented in Fig. 2 (total protein) with Fig. 4 (cellular fractions). First, samples in Fig. 2a are crude total protein homogenates prepared in the absence of protease inhibitors, whereas fractionated samples in Fig. 4 were further purified through high-speed ultracentrifugation and in the presence of protease inhibitors. Second, to visualise comparative expression across brain regions and ages in fractionated samples (Fig. 4), equivalent sample loads for nuclear and membrane fraction were loaded resulting in an oversaturation of signal particularly in parietal cortex. Thus, caution should be exercised with regard to interpretation of expression level of full-length α_3 and α_{3N}, particularly in cortex at 100 days GA.

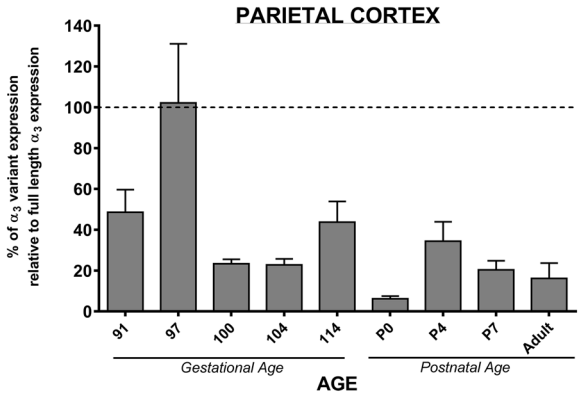
Immunoprecipitation experiments were performed to verify the specificity of the variant band and to investigate interactions with other $GABA_A$ receptor subunits and the neuronal assembly protein gephyrin. Immunoprecipitation of pig brain lysates was performed for $GABA_A$ receptor subunits α_3, α_1, γ_2, and gephyrin, and recovered immunocomplexes interrogated using western blot. Western blot analysis of $GABA_A$ receptor α_1, γ_2, and gephyrin immunoprecipitates with anti-$GABA_A$ receptor α_3 revealed a direct association of both the full-length α_3 (55 kDa) and variant α_{3N} (49 kDa) proteins with the $GABA_A$ receptor subunits α_1 and γ_2, but not with gephyrin. To confirm this association, the reverse immunoblots were performed with anti-$GABA_A$ receptor α_1 and anti-$GABA_A$ receptor γ_2 on the $GABA_A$ receptor α_3 immunoprecipitate which showed a corresponding band for α_1 (51 kDa) and γ_2 (44–46 kDa) (Fig. 5). No direct association of the $GABA_A$ receptor α_3, α_{3N}, or α_1 subunits was found with gephyrin; however, the western blot with anti-γ_2 show an interaction with gephyrin in pig brain.

Discussion

The aim of this study was to identify an unknown protein in western blots that routinely reacted to the $GABA_A$ receptor α_3 subunit antibody in brain tissue from the neonatal pig (Kalanjati et al. 2011; Miller et al. 2016, 2017). PCR and sequencing analysis revealed this protein to be a splice variant of the $GABA_A$ receptor α_3 subunit. However, unlike other reported splice variants of the $GABA_A$ receptor, this splice variant appears unique in that it is preferentially expressed in the developing brain. Several groups have previously observed this unknown second band in the developing brain. McKernan et al. reported the full-length $GABA_A$ receptor α_3 subunit as well as inconsistent expression of a smaller band that reacted to a α_3-specific antibody in the rat cortex from birth through to adulthood—this antibody was directed toward the cytoplasmic loop of the $GABA_A$ receptor (McKernan et al. 1991). In cat visual cortex, Chen et al. also described a smaller band immunoreactive to a α_3-specific antibody (directed toward the same N-terminal region as the Merck-Millipore antibody) in the 1-week-old cat which disappeared by 5 weeks of age (Chen et al. 2001). Both groups concluded that this smaller band was likely the result of proteolytic cleavage, posttranslational modification, or a developmentally specific isoform. Two splice variants of the $GABA_A$ receptor α_3 subunit are registered in the NCBI database (XM_006724811.2 and XM_013047929.1); however, these are only predicted sequences generated through automated computational analysis. Alignment of the pig α_{3N} variant with the predicted sequence XM_013047929.1 showed 95% sequence similarity at the protein level, suggesting that the latter is the human orthologue. Our study is the first to report a $GABA_A$ receptor α_3 subunit splice variant in native brain tissue.

The $GABA_A$ receptor belongs to the cys-loop ligand-gated ion channel family of receptors the characteristics of which are a receptor comprised of five subunits that form a central pore. Subunits from this family have a structure consisting of a large extracellular N-terminal region and four TM domains (TM1–TM4) with a large intracellular loop between TM3 and TM4 (Fig. 1b) (Sine and Engel 2006). The TM2 is an important part of the $GABA_A$ receptor with many of its residues forming the interior of the channel pore and thus is essential for determining ion selectivity (Sine and Engel 2006). The removal of exon 8 would have a significant effect on the subunit structure as exon 8 spans the region containing all of TM1 and terminates seven amino acids into TM2. Alternative splicing of several $GABA_A$ receptor subunits exists; however, these splice sites occur within the large intracellular loop between TM3 and TM4 or in


FRONTAL CORTEX
% of α_3 variant expression relative to full length α_3 expression
120
100
80
60
40
20
0
91
97
100
104
114
P0
P4
P7
Adult
Gestational Age
Postnatal Age
AGE


PARIETAL CORTEX
% of α_3 variant expression relative to full length α_3 expression
140
120
100
80
60
40
20
0
91
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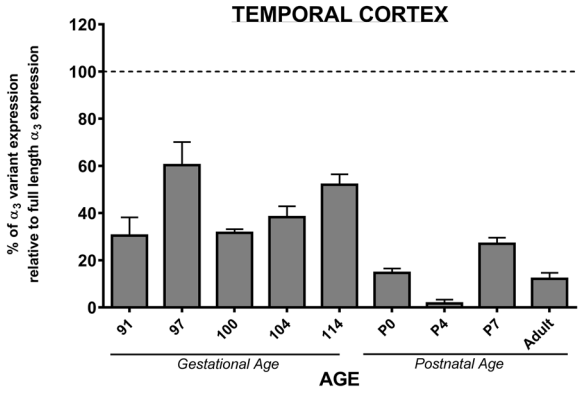
100
104
114
P0
P4
P7
Adult
Gestational Age
Postnatal Age
AGE


TEMPORAL CORTEX
% of α_3 variant expression relative to full length α_3 expression
120
100
80
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40
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0
91
97
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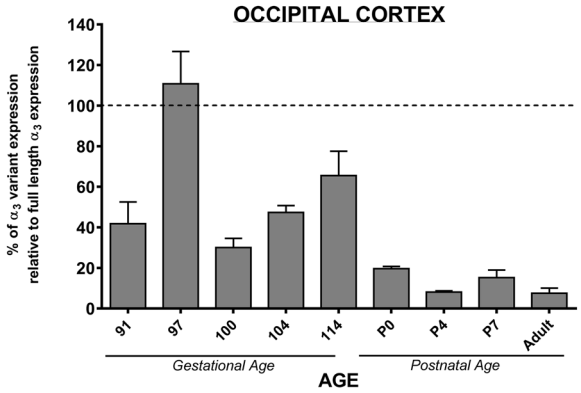
114
P0
P4
P7
Adult
Gestational Age
Postnatal Age
AGE


OCCIPITAL CORTEX
% of α_3 variant expression relative to full length α_3 expression
140
120
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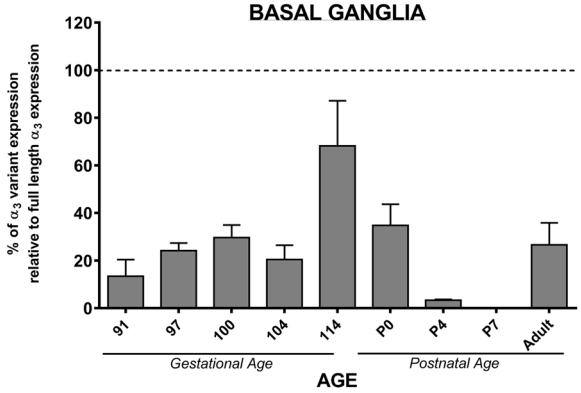
Adult
Gestational Age
Postnatal Age
AGE


BASAL GANGLIA
% of α_3 variant expression relative to full length α_3 expression
120
100
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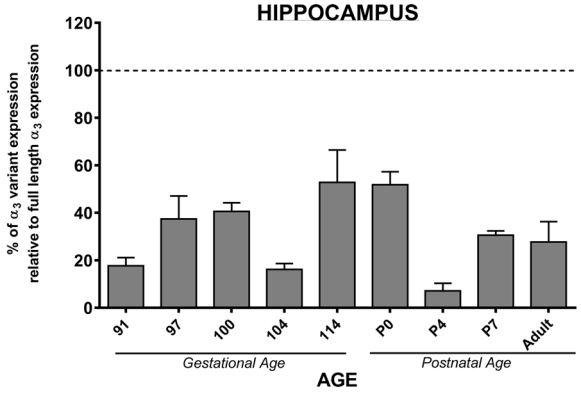
P0
P4
P7
Adult
Gestational Age
Postnatal Age
AGE


HIPPOCAMPUS
% of α_3 variant expression relative to full length α_3 expression
120
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P0
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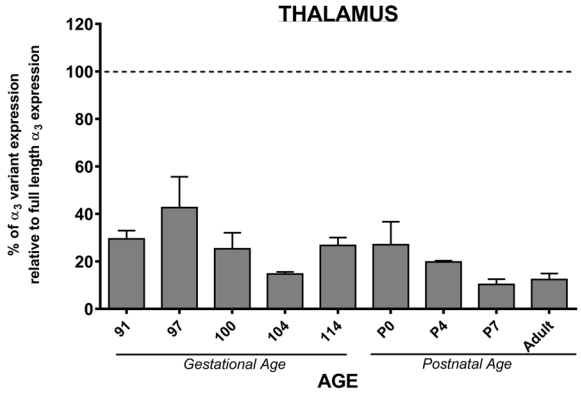
P7
Adult
Gestational Age
Postnatal Age
AGE


THALAMUS
% of α_3 variant expression relative to full length α_3 expression
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P4
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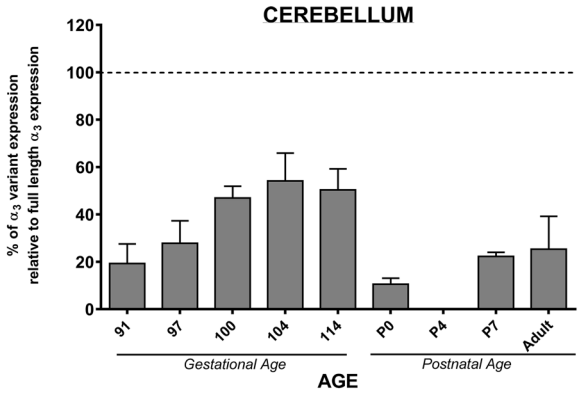
Adult
Gestational Age
Postnatal Age
AGE


CEREBELLUM
% of α_3 variant expression relative to full length α_3 expression
120
100
80
60
40
20
0
91
97
100
104
114
P0
P4
P7
Adult
Gestational Age
Postnatal Age
AGE

◂**Fig. 3** Expression of the $GABA_A$ receptor α_{3N} variant as a percentage of full-length $GABA_A$ receptor α_3 protein expression at each developmental age. Dotted line representative of full-length $GABA_A$ α_3 expression at each age. The $GABA_A$ receptor α_{3N} variant displayed temporal expression changes as well as differential expression across several brain regions. In cortical regions, α_{3N} expression was highly expressed during early development decreasing to virtually negligible levels by adulthood. In the hippocampus α_3 variant expression relative to full-length α_3, varied with age. In the basal ganglia, thalamus and cerebellum α_3 variant expression remained ubiquitously low, while full-length α_3 expression decreased resulting in moderate alterations in the α_3 variant expression relative to full-length expression with age

extracellular N-terminal sites (Alger and Möhler 2001). N-terminal sequences of $GABA_A$ receptor subunits are the principal sites for subunit–subunit interaction and oligomerisation, while the intracellular loop between TM3 and TM4 allows for interactions with cytosolic regulatory proteins which are important for the regulation of receptor trafficking, localisation, and activity (Arancibia-Carcamo and Kittler 2009).

Multiple variants of a gene are extremely common, but can result in proteins that are either functionally undifferentiated or non-functional (Johnson et al. 2003). Cellular fractionation results reported in our current study revealed differential expression of the $GABA_A$ receptor α_{3N} variant across gestational age suggesting a developmentally specific role for this splice variant. Furthermore, expression of the variant was observed in the membrane fraction of all brain regions as well as an interaction with other $GABA_A$ receptor subunits suggestive of a functional role. In fetal piglet brain (91 and 100 day GA), the $GABA_A$ receptor α_{3N} variant was highly abundant in the membrane and nuclear fractions of the three brain regions investigated, but was expressed at much lower levels in postnatal brain membrane and nuclear fractions relative to the full-length $GABA_A$ receptor α_3 protein. While this is particularly evident in hippocampus and thalamus, this does not appear to be the case in parietal cortex due to much higher overall expression. It must be noted that to visualise the comparative expression across brain regions and ages (Fig. 4), equivalent sample loads for nuclear and membrane fraction (5 µg) were loaded resulting in an oversaturation of signal particularly in parietal cortex. Optimisation of antibody concentration to within the linear dynamic range of film for quantitation (data not shown) resulted in significantly, if not absent, expression of the variant at postnatal ages in parietal cortex and hippocampus. Cytosolic expression of the α_3 and α_{3N} variants was much lower compared with membrane and nuclear fractions requiring much higher sample loads (20 µg) to visualise expression. However, there was a striking age-dependant shift in cytosolic expression from full-length α_3 expression in the fetal brain to α_{3N} variant expression in postnatal brain. $GABA_A$ receptors are not static entities on the plasma membrane, but are believed to cycle continuously between intracellular compartments and the cell surface (Vithlani et al. 2011). A small cytoplasmic pool of α_{3N} variant was evident; however, it is not known whether this residual protein may be targeted for degradation or may play a regulatory role in expression of the full-length α_3 protein in the plasma membrane (Vallejo-Illarramendi et al. 2005). The fact that cytosolic expression switches, however, suggests that α_{3N} may have a functional role during brain development.

The $GABA_A$ receptor α_3 protein dominates during fetal brain development up until birth, where the α_1 protein takes over as the major α-subunit in the adult brain (Miller et al. 2017). While $GABA_A$ receptor function is mediated by the subunit composition of the receptor, individual $GABA_A$ receptor subunits also participate in post-synaptic targeting and clustering of receptor complexes to the membrane (Jacob et al. 2009; Luscher et al. 2011). The γ_2 and δ subunits are known to have key roles in $GABA_A$ receptor targeting (Alldred et al. 2005; Essrich et al. 1998; Wu et al. 2012). However, the α-subunit has also recently been reported to have a key role in $GABA_A$ receptor assembly and clustering of receptors to synaptic sites (Vithlani et al. 2011; Wu et al. 2012). The α_2 and α_6 subunits show differential function in the guiding of $GABA_A$ receptors to synaptic and extrasynaptic locations, respectively (Wu et al. 2012). Furthermore, the α-subunit has been reported to have a critical role in $GABA_A$ receptor targeting to the membrane through its interaction with the scaffold protein gephyrin (Wu et al. 2012). In transfection studies, a direct interaction of gephyrin has been reported with the α_1 (Mukherjee et al. 2011), α_2 (Saiepour et al. 2010), and α_3 subunits (Tretter et al. 2011). In our IP experiments in pig neonatal brain tissue, we did not show an interaction between gephyrin and either the α_1 or α_3 subunits; however, we did confirm a direct interaction of gephyrin with the γ_2-subunit. The lack of interaction of the α-subunits with gephyrin appears at odds with findings from the previous studies using cultured and transfected neurones; however, caution should be taken in inferring in vivo interactions from in vitro work. Transfection studies where there is an over-expression of the protein of interest may 'force' an interaction which *in vivo* is not evident. While co-localisation of gephyrin with α_1 and α_3 subunits has been observed *in vivo* in immunolabelling studies in the rat brain (Gross et al. 2011; Sassoe-Pognetto et al. 2000), to the best of our knowledge a direct interaction of α-subunits with gephyrin has not been demonstrated by IP in native tissue.

The unique presence of this variant in neonatal brain suggests an important role in the development and functioning of the immature excitatory $GABA_A$ receptor system and its trophic role in development. Studies to further characterise this splice variant and its function are warranted.

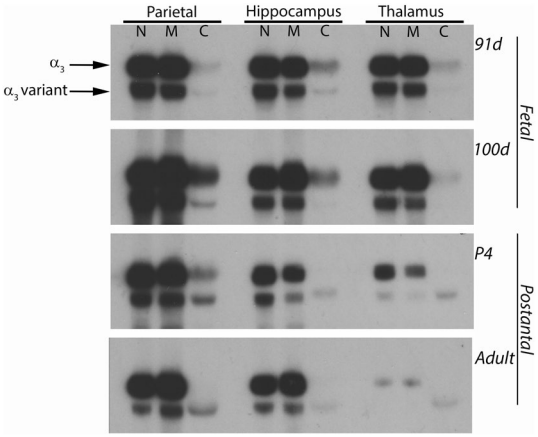


Fig. 4 Cellular localisation of $GABA_A$ receptor α_3 protein and α_{3N} variant. Parietal cortex, hippocampus, and thalamus samples from two fetal ages (91 and 100 days GA) and two postnatal ages (P4 and adult) were fractionated to obtain the nuclear, cytoplasmic, and membrane fractions. Western blot analysis revealed localisation of both the full-length $GABA_A$ receptor α_3 protein and α_{3N} variant to nuclear and membrane fractions, although α_{3N} variant expression was higher in developing fetal brain compared with postnatal ages. Cytosolic expression of both proteins was much lower; however, there was a distinct switch in abundance of the α_3 and α_{3N} variant expression with age. Full-length cytosolic $GABA_A$ receptor α_3 was expressed almost exclusively in fetal brain while the α_{3N} variant was exclusively expressed in the postnatal brain. Parietal cortex showed overlap of α_3 and α_{3N} expression closer to birth, but by adulthood, only the α_{3N} variant was expressed in the cytosol. N = Nuclear (5 µg), M = membrane (5 µg), C = cytosolic (20 µg)

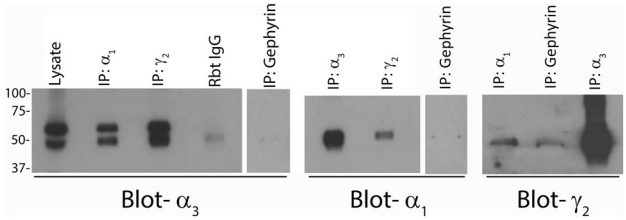


Fig. 5 Immunoprecipitation of pig brain lysates with $GABA_A$ receptor subunit α_3, α_1, γ_2, and gephyrin antibodies. Captured immunocomplexes were subjected to western blot analysis and probed with the same antibodies. Immunoblots with anti-$GABA_A$ receptor α_3 (Blot-α_3) show co-precipitation of both full-length α_3 (55 kDa) and variant α_{3N} (49 kDa) proteins with $GABA_A$ receptor subunits α_1 and γ_2, but not with gephyrin. Reverse immunoblots with anti-$GABA_A$ receptor α_1 (Blot-α_1) and anti-$GABA_A$ receptor γ_2 (Blot-γ_2) confirmed this interaction. Gephyrin was found to interact only with $GABA_A$ receptor γ_2 in pig brain

Acknowledgements Financial support was provided by The National Health and Medical Research Council (Australia) (Grant ID: 569826 and 351501). STB is supported by a Lion's Medical Research Foundation Senior Fellowship. PBC is supported by an NHMRC Practitioner Fellowship. VPK is a recipient of an Australian Development Scholarship. Thanks to Morgan Apel and Jack Kibble for technical assistance.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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