DOCTOR OF PHILOSOPHY

Endogenous neurosteroid actions at GABAA receptors during neuronal development

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ABBREVIATIONS

$\alpha1^{0/0}$	$GABA_AR$ α1 subunit "knockout"
$\alpha4^{0/0}$	$GABA_AR$ α4 subunit "knockout"
$\delta^{0/0}$	$GABA_AR$ δ subunit "knockout"
3α-HSD	3α-hydroxysteroiddehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
5α3α	5α-pregnan-3α-ol-20-one
5α3β	5α-pregnan-3β-ol-20-one
5β3α	5β-pregnan-3α-ol-20-one
5β3β	5β-pregnan-3β-ol-20-one
5α-THDOC	5α-pregnan-3α,21-diol-20-one or 5α-tetrahydrodeoxycorticosterone
$5\text{-}HT_3$	5-hydroxytryptamine type 3 receptor
5α-DHP	5α-dihydroprogesterone or 5α-pregnane-3,20-dione
5α-R	5α-reductase
α-CD	α-cyclodextrin
β-CD	β-cyclodextrin
γ-CD	γ-cyclodextrin
γ2S	Short splice variant of $GABA_A$ receptor γ_2-subunit
γ2L	Long splice variant of $GABA_A$ receptor γ_2-subunit
τ_w	Weighted decay time constant of mIPSC decay
aCSF	Artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid type glutamate receptor
ANOVA	Analysis of variance statistical test
ATP	Adenosine triphosphate
BGT-1	Betaine/GABA transporter
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
CaMKII	Ca^{2+}-calmoduline-dependent protein kinase II
CGC	Cerebellar granule cell
CNS	Central nervous system
DGGC	Dentate gyrus granule cell
DHEA	Dehydroepiandrosterone
dLGN	Dorsolateral geniculate nucleus of the thalamus

DMCM	6,7,dimethoxy-4-ethyl-β-carboline-3-carboxylic acid Methyl ester
DMSO	Dimethylsulphoxide
DRG	Dorsal root ganglion
DS2	4-chloro-N-[2-(2-thienyl)imidazo[1,2-a]pyridine-3-yl benzamide
EC_{50}	The concentration of compound producing 50% of the maximal effect
E_{Cl^-}	Reversal potential for chloride ions
ECS	Extracellular solution
EGTA	Ethylene glycol-bis(β-aminoethyl-ether)-N,N,N'N' tetracetic acid
eIPSC	Evoked inhibitory postsynaptic current
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
GABA	γ-aminobutyric acid
$GABA_AR$	γ-aminobutyric acid type A receptor
$GABA_BR$	γ-aminobutyric acid type B receptor
$GABA_CR$	γ-aminobutyric acid type C receptor
GABA-T	GABA transaminase
GAD	Glutamic acid decarboxylase
GAE	Generalised absence epilepsy
GFP	Green fluorescent protein
GAT_{1-4}	GABA transporter types 1-4
HEPES	N-2-Hydroxyethylpiperazine-N'2-ethanesulphonic acid
IEI	Inter-event interval
IPSC	Inhibitory postsynaptic current
IPSP	Inhibitory postsynaptic potential
I-V	Current-voltage
KS	Kolmogorov-Smirnov statistical test
LORR	Loss of righting reflex
LTS	Low threshold spike
L1	Layer 1 of the neocortex
L2/3	Layer 2 and 3 of the neocortex
L4	Layer 4 of the neocortex

L5	Layer 5 of the neocortex
L6	Layer 6 of the neocortex
mIPSC	Miniature inhibitory postsynaptic current
mEPSC	Miniature excitatory postsynaptic current
mRNA	Messenger ribonucleic acid
NMDA	N-methyl-D-aspartate type glutamate receptor
nACh	Nicotinic acetylcholine receptor
nRT	*nucleus reticularis* of the thalamus
P	Postnatal day
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PKC_{ε}	Protein kinase C, epsilon isoform
PNS	Peripheral nervous system
PPD	Post-partum dysphoria
PREG	Pregnenolone
PROG	Progesterone
$P450_{SCC}$	Cytochrome P450 cholesterol side-chain cleavage
RM ANOVA	Repeated measures ANOVA statistical test
RT-PCR	Reverse transcription-polymerase chain reaction
SEM	Standard error of the mean
SON	Supraoptic nucleus of the hypothalamus
SSRI	Selective serotonin re-uptake inhibitor
sIPSC	Spontaneous inhibitory postsynaptic current
T50	Time taken for mIPSCs to decay by 50%
T90	Time taken for mIPSCs to decay by 90%
TBPS	*tert*-butylbicyclophosphorothionate
THIP	4,5,6,7-tetraydroisoxazol[5,4-c]pyridin-3-ol
TM1-4	Transmembrane spanning domains 1-4
TSPO	18kDatranslocater protein
TTX	Tetrodotoxin
VB	*Ventrobasal* nucleus of the thalamus
VDCC	Voltage-dependent Ca^{2+} channel
VGAT	Vesicular GABA transporter
WT	Wild type
ZAC	Zinc activated ion channel

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CANDIDATE'S DECLARATION

I declare that I am the author of this thesis and that it is a true record of the work performed by me. This thesis has not been previously submitted for application for a higher degree. All sources of information used in the preparation of this thesis have been consulted and are cited correctly. This work has been carried out in the Centre for Neuroscience, Division of Medical Sciences, University of Dundee, under the supervision of Dr. Delia Belelli, Prof. Jeremy J. Lambert and Prof. David J. K. Balfour.

Adam Brown

SUPERVISOR'S DECLARATION

I certify that Adam R. Brown has completed nine terms of experimental research and has fulfilled the conditions of Ordinance 39, University of Dundee, such that he is eligible to submit the following thesis in application for the degree of Doctor of Philosophy.

Professor J.J. Lambert

Dr D. Belelli

Professor D.J.K. Balfour

ABSTRACT

$GABA_A$ receptors ($GABA_ARs$) mediate the majority of fast inhibition in the CNS and as such are crucial to neuronal function. Two distinct modes of $GABA_AR$ mediated inhibition exist: "phasic" involves the transient activation of postsynaptic $GABA_ARs$ following presynaptic vesicular GABA release and "tonic" whereby high-affinity extrasynaptic $GABA_ARs$ are persistently activated by ambient GABA. $GABA_ARs$ exhibit a rich pharmacology and are the target for a number of clinically useful compounds including benzodiazepines, barbiturates and certain general anaesthetics. In addition, several naturally occurring steroids typified by the progesterone metabolite 5α-pregnan-3α-ol-20-one (5α3α) are potent positive allosteric modulators of $GABA_AR$ function - a property that endows these steroids with anxiolytic, sedative and anti-convulsant actions. Importantly, in addition to importing steroids from the periphery, the brain also harbours a steroidogenic capacity and can manufacture GABA-modulatory steroids, termed "neurosteroids", from cholesterol. Although several studies have demonstrated neurosteroids to be implicated in a variety of physiological processes including neurodevelopment, important questions remain. In particular, are neurosteroids neurone selective? Do they discriminate between particular neuronal $GABA_ARs$ subtypes? Under what conditions do endogenous neurosteroids influence neuronal function? And importantly, which cells synthesise neurosteroids in the central nervous system?

Therefore, the principal aims of this study were: 1) to characterise the properties of $GABA_AR$ mediated inhibition in postnatal day (P) 17-24 neurones of the mouse ventrobasal (VB) nucleus of the thalamus – neurones which have a well defined $GABA_AR$ expression profile and which exhibit both synaptic and tonic $GABA_AR$ mediated inhibition. 2) To investigate the effects of 5α3α on synaptic and tonic $GABA_AR$ mediated inhibition in VB neurones. 3) To investigate whether there is a role for endogenous neurosteroids in regulating synaptic $GABA_AR$ function during postnatal

development (P7-P20) in VB neurones and 4) cortical layer 2/3 neurones – a neuronal population with a more heterogenous, and less well defined $GABA_AR$ expression profile.

To address these aims, whole-cell voltage clamp recordings were performed on acute brain slices derived from wild type and transgenic $GABA_AR$ subunit "knock-out" mice in conjunction with pharmacological approaches. Synaptic inhibition in WT VB neurones, as inferred by recording miniature inhibitory postsynaptic currents (mIPSCs) was characterised by relatively large amplitude and fast decaying mIPSCs. A large tonic conductance in WT VB neurones was inhibited following application of the competitive $GABA_AR$ antagonist bicuculline (30 μM). Deletion of the α4 subunit ($\alpha4^{0/0}$) revealed alteration to both synaptic and tonic inhibition. Most notably, $\alpha4^{0/0}$ VB neurones displayed a greatly diminished tonic conductance. These results are in agreement that the majority of extrasynaptic $GABA_ARs$ in VB neurones contain the α4 subunit. Exogenous application of the neurosteroid 5α3α (100 nM) to WT VB neurones only modestly enhanced the tonic conductance and also gave rise to mIPSCs with prolonged decay kinetics. It is concluded 5α3α has a relatively low potency at the extrasynaptic $GABA_AR$ population in VB neurones and furthermore that this concentration does not discriminate between synaptic and tonic inhibition.

Between P7 and P20, mIPSCs recorded from VB and L2/3 neurones became progressively faster decaying, a feature that has been associated with an increase in α1-$GABA_AR$ subunit expression during development. However, the developmental decrease in mIPSC decay time was still observed in recordings from $\alpha1^{0/0}$ L2/3 pyramidal neurones indicating the contribution of additional factors. Here I show that blocking neurosteroid synthesis using the 5α-reductase inhibitor, finasteride, or treating the brain slice with the steroid-scavenger molecule, γ-cyclodextrin (γ-CD), results in significantly faster mIPSC decay times between P7 and P10 in VB and L2/3 neurones.

These results provide the first indication that an endogenous neurosteroid tone may influence the mIPSC decay kinetics of VB and L2/3 neurones during development. Moreover, compared to γ-CD pre-incubation, application of γ-CD to the intracellular compartment *via* the patch pipette was found to be equally effective thus suggesting a possible autocrine mechanism of neurosteroid action. For VB neurones at P20-24 (an age range at which the mIPSCs are insensitive to γ-CD treatment), incubation with the $GABA_AR$-inactive neurosteroid precursor 5α-DHP resulted in a robust prolongation of the mIPSC decay time thus revealing the presence of functional neurosteroidogenic enzymes in the brain slice tissue.

In summary, these results reveal for the first time that during the first ~2 weeks of life the developmental decrease in the duration of the mIPSCs recorded from VB and L2/3 neurones is largely due to a reduction in local neurosteroid synthesis. Moreover, P20-24 VB neurones are capable of reinitiating neurosteroid production thus giving rise to a mechanism that may, under certain conditions, locally influence neuronal inhibition. These results may have important physiological consequences as the developmental timing of the neurosteroid tone observed here coincides with a myriad of crucial neurodevelopmental processes including the transition of GABA from a depolarising to a hyperpolarising action.

AIMS

The aims of this project were:

- To characterise the properties of both the mIPSCs (mediated by synaptic $GABA_A$Rs) and the tonic conductance (mediated by extrasynaptic $GABA_A$Rs) in VB neurones. These studies were performed using the whole cell patch clamp technique applied to *in vitro* brain slices prepared from P17-24 wild type (WT) and $\alpha4^{0/0}$ mice.

- To compare the effects of DS2, THIP and the neurosteroid 5α3α on synaptic *vs*extrasynaptic $GABA_A$Rs in P17-24 VB neurones derived from WT and $GABA_A$R subunit "knock-out" mice.

- To investigate the possibility that endogenous neurosteroid levels regulate synaptic $GABA_A$R function during postnatal development (P7-P20) in VB neurones.

- To investigate the possibility that endogenous neurosteroid levels regulate synaptic $GABA_A$R function during postnatal development (P7-P20) in cortical layer L2/3 pyramidal neurones and L2/3 cortical interneurones.