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Realising the therapeutic potential of neuroactive steroid modulators of the $GABA_A$ receptor

Delia Belelli[a], Derk Hogenkamp[b], Kelvin W. Gee[b], Jeremy J. Lambert[a,*]

[a] Systems Medicine, Neuroscience, Mail Box 6, University of Dundee, Ninewells Hospital and Medical School, Dundee, DD1 9SY, United Kingdom
[b] Department of Pharmacology, 110C Med Surge1, Mail Code 4625, University of California, Irvine, School of Medicine, Irvine, CA, 92697, USA

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ABSTRACT

In the 1980s particular endogenous metabolites of progesterone and of deoxycorticosterone were revealed to be potent, efficacious, positive allosteric modulators (PAMs) of the $GABA_A$ receptor ($GABA_AR$). These reports were followed by the discovery that such steroids may be synthesised not only in peripheral endocrine glands, but locally in the central nervous system (CNS), to potentially act as paracrine, or autocrine "neurosteroid" messengers, thereby fine tuning neuronal inhibition. These discoveries triggered enthusiasm to elucidate the physiological role of such neurosteroids and explore whether their levels may be perturbed in particular psychiatric and neurological disorders. In preclinical studies the $GABA_AR$-active steroids were shown to exhibit anxiolytic, anticonvulsant, analgesic and sedative properties and at relatively high doses to induce a state of general anaesthesia. Collectively, these findings encouraged efforts to investigate the therapeutic potential of neurosteroids and related synthetic analogues. However, following over 30 years of investigation, realising their possible medical potential has proved challenging. The recent FDA approval for the natural neurosteroid allopregnanolone (brexanolone) to treat postpartum depression (PPD) should trigger renewed enthusiasm for neurosteroid research. Here we focus on the influence of neuroactive steroids on GABA-ergic signalling and on the challenges faced in developing such steroids as anaesthetics, sedatives, analgesics, anticonvulsants, antidepressants and as treatments for neurodegenerative disorders.

1. Introduction

Almost 80 years ago Hans Selye demonstrated that particular pregnane steroids can induce rapid sedation and a state of unconsciousness (Selye, 1941). Approximately 40 years elapsed before a viable molecular mechanism emerged to explain these rapid behavioural effects. Electrophysiological studies revealed the intravenous synthetic steroidal general anesthetic alphaxalone (5α-pregnane-3α-ol-11, 20-dione) to enhance the inhibitory effects of GABA in guinea pig olfactory cortex (Scholfield, 1980) and in cat spinal cord neurons (Lodge and Anis, 1984). Extracellular recordings in the rat cuneate nucleus demonstrated alphaxalone to potently enhance responses mediated by $GABA_A$ receptor ($GABA_AR$) agonists (Harrison and Simmonds, 1984). Supporting the $GABA_AR$ as a locus for the behavioural effect, betaxalone, a 3β-ol isomer of alphaxalone, had no effect on $GABA_ARs$ and was behaviourally inert (Harrison and Simmonds, 1984). In their seminal paper they recalled that alphaxalone was structurally related to certain endogenous pregnane steroids, raising the prospect that the activity of the major inhibitory receptor in the mammalian central nervous system (CNS), the $GABA_AR$, may be subject to physiological, or pathophysiological regulation (Harrison and Simmonds, 1984). In confirmation, subsequent electrophysiological and radioligand binding approaches revealed particular metabolites of progesterone (*e.g.*, 5α-pregnan-3α-ol-20-one, also known as allopregnanolone and now brexanolone) and of deoxycorticosterone (5α-pregnan-3α, 21-diol-20-one, [5α-THDOC]) to also be highly efficacious, stereoselective, positive allosteric modulators (PAMs) of the $GABA_AR$ at nM concentrations (Majewska et al., 1986; Barker et al., 1987; Callachan et al., 1987; Cottrell et al., 1987; Gee et al., 1988; Peters et al., 1988). Radioligand binding and electrophysiological studies established that the GABA facilitatory actions of these steroids were not mediated by binding to the proposed benzodiazepine site on the $GABA_AR$ (Harrison and Simmonds, 1984; Cottrell et al., 1987; Gee et al., 1988). In common with barbiturates, these steroids promoted the open conducting state of the $GABA_AR$ associated anion channel, thereby enhancing the actions of GABA and at higher concentrations, they directly activated the receptor (Majewska et al., 1986; Barker et al., 1987; Callachan et al., 1987; Cottrell et al., 1987; Peters et al., 1988), leading

* Corresponding author.
E-mail address: j.j.lambert@dundee.ac.uk (J.J. Lambert).

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Abbreviations

Ad	Alzheimer's disease
allopregnanolone	5α-pregnan-3α-ol-20-one
CNS	central nervous system
DGGCs	dentate gyrus granule cells
EEG	electroencephalogram
FDA	food and drug administration
$GABA_AR$	$GABA_A$ receptor
HPA	hypothalamus, pituitary, adrenal axis
IPSC	inhibitory postsynaptic current
im	intramuscular
iv	intravenous
MOA	mechanism of action
nRT	nucleus reticularis
NREM	non rapid eye movement
ORG	ORG OD02-0
PAM	positive allosteric modulator
PD	pharmacodynamic
pGPCR	progesterone G-protein coupled receptor
PK	pharmacokinetic
PKA	protein kinase A
PKC	protein kinase C
po	oral administration
PPD	postpartum depression
REM	rapid eye movement
SE	status epilepticus
VB	ventrobasal

to their description as "barbiturate-like" modulators (Harrison and Simmonds, 1984 Majewska et al., 1986), although subsequently barbiturate/steroid interaction studies implied these $GABA_AR$ modulators acted *via* distinct sites (Cottrell et al., 1987; Gee et al., 1988; Peters et al., 1988). The potency and stereoselectivity of their actions suggested involvement of a high affinity steroid binding site located on the $GABA_AR$. However, the apparent affinity derived from functional studies may be misleading as these steroids are highly lipophilic, causing their accumulation to relatively high local concentrations in the membrane *i.e.* in close apposition to a proposed transmembrane binding site for the steroid on the $GABA_AR$ protein (Hosie et al., 2006; Chisari et al., 2010).

In this review we use the term neurosteroid for endogenous steroids synthesised in the CNS and the term neuroactive steroid for exogenous $GABA_AR$-active steroids.

2. Neuroactive steroids and $GABA_AR$ subtypes

The $GABA_AR$ is a member of the cys-loop transmitter-gated ion channel family and is composed of five transmembrane crossing subunits. Studies in the late 1980s and early 1990s revealed an unexpected diversity of $GABA_AR$ subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π and ρ1-3), a repertoire supporting the expression of ~20–30 subtypes of $GABA_AR$ (Olsen and Sieghart, 2009), that exhibit a distinct expression pattern within the mammalian CNS. The majority of $GABA_ARs$ are composed of two α subunits, two β subunits and a γ subunit (predominantly, but not exclusively the γ2 subunit). For some receptors the δ subunit (δ-$GABA_ARs$) replaces the γ subunit. The $GABA_AR$ subunit composition influences *i)* where in the CNS the receptors are expressed and their subcellular location within a neuron *e.g.* synaptic *vs* extra/peri-synaptic expression *ii)* the biophysical properties of the receptor, including rates of deactivation, desensitization, and GABA affinity *iii*) the effect of enzymes such as kinases and phosphatases upon receptor function and expression), and *iv)* receptor pharmacology (Jacob et al., 2008; Olsen and Sieghart, 2008; Fritschy and Panzanelli, 2014; Nakamura et al., 2015).

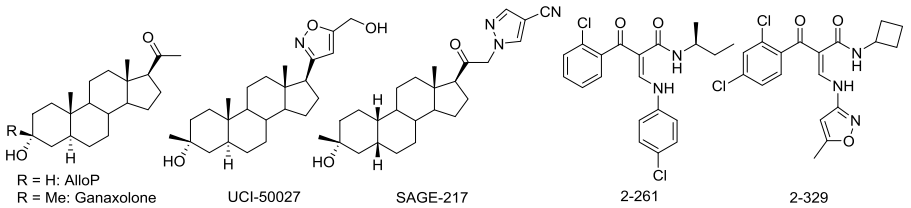
In common with established PAMs of the $GABA_AR$ (*e.g.* diazepam), neuroactive steroids exhibit anxiolytic, analgesic, anticonvulsant and sedative effects, but additionally at greater doses they induce a state of general anaesthesia (Belelli and Lambert, 2005; Reddy and Estes, 2016). Although such steroids are highly selective PAMs of the $GABA_AR$, they exhibit only limited specificity for particular $GABA_AR$ isoforms (Belelli et al., 2009 Brickley and Mody, 2012). A possible exception are δ-$GABA_ARs$, where neurosteroids appear highly efficacious, although this apparent selectivity is probably secondary to the limited efficacy of GABA acting at δ-$GABA_ARs$ (Brickley and Mody, 2012). Table 1 utilises the voltage-clamp technique to compare the $GABA_AR$ enhancing effects of allopregnanolone, with structurally related synthetic steroids across representatives of synaptic and extrasynaptic $GABA_ARs$. In agreement with previous studies (Belelli et al., 2009 Brickley and Mody, 2012) inspection of Table 1 reveals little $GABA_AR$ isoform specificity of these steroids. Given this $GABA_AR$ promiscuity a key question concerns the identification of the $GABA_AR$ subtypes that mediate the behavioural repertoire neurosteroids. Experiments with "knock-in" mice genetically engineered to express diazepam-insensitive and etomidate-insensitive $GABA_ARs$ have revealed components of their behavioural effects to be mediated by particular $GABA_ARs$ (Rudolph and Knoflach, 2011). Key transmembrane amino acids have been identified that impair neurosteroid modulation of $GABA_ARs$, permitting equivalent mouse behavioural studies, although to date there has not been a comprehensive behavioural profile of such mice (Newman et al., 2016). In the absence of such studies, components of the behavioural effects neuroactive steroids may exhibit a similar receptor profile to those of other $GABA_AR$ modulators. However, whereas the effects of diazepam are mediated by enhancing the effects of GABA at γ-$GABA_ARs$, importantly neurosteroids additionally act as PAMs of δ-$GABA_ARs$. Indeed, behavioural studies of mice with deletion of the genes encoding for the δ subunit and the α4 subunit (often partnered with the δ subunit) are proving instructive (see below).

Receptors incorporating the γ2 subunit, are primarily, although not exclusively, expressed within inhibitory synapses, where they mediate fast phasic responses (under voltage-clamp conditions recorded as inhibitory postsynaptic currents [IPSCs]) upon activation by the neurally-evoked vesicular release of GABA (Farrant and Nusser, 2005; Brickley and Mody, 2012). In common, both benzodiazepines such as diazepam and neuroactive steroids prolong the IPSC duration, thereby enhancing phasic inhibition. Certain neurons (*e.g.* hippocampal dentate gyrus granule cells [DGGCs]; thalamocortical ventrobasal [VB] neurons) additionally express neurosteroid-sensitive δ-$GABA_ARs$ (Brickley and Mody, 2012). These receptors are insensitive to benzodiazepines and are predominantly expressed out with the inhibitory synapse in perisynaptic, or extrasynaptic locations (Brickley and Mody, 2012). In these neurons extrasynaptic δ-$GABA_ARs$ mediate a tonic, persistent current, either in response to low ambient levels of GABA, or due to spontaneous gating of the receptor-channel complex (Brickley and Mody, 2012; Wlodarczyk et al., 2013). Neuroactive steroids, in common with $GABA_AR$-active general anaesthetics, such as etomidate and propofol, will interact with synaptic γ2-$GABA_ARs$ to prolong the duration of phasic inhibition, but additionally with extrasynaptic δ-$GABA_ARs$ to increase tonic inhibition (Stell et al., 2003; Belelli and Lambert, 2005; Peden et al., 2008; Brickley and Mody, 2012) – Fig. 1. By contrast, benzodiazepines will primarily influence phasic inhibition, although certain neurons do express benzodiazepine-sensitive γ2 subunit containing $GABA_ARs$ (*e.g.* α5βγ2), that mediate a tonic conductance (Caraiscos et al., 2004);

Considering how neurons respond during physiological rates of presynaptic stimulation, this division of fast phasic and tonic inhibition may be an over simplification. For example thalamocortical VB neurons

Table 1

The potency and maximal efficacy of neuroactive steroids and non-steroidal modulators on the function of human $GABA_AR$ subtypes expressed in *Xenopus laevis* oocytes. The GABA EC_{10} (the concentration of GABA that evokes a response 10% of the maximal response to GABA) was determined for each oocyte expressing the receptor subtype of interest All compounds were tested with a 30 s pre-treatment prior to co-application with an EC_{10} concentration of GABA. The effect of the test drug upon the GABA EC_{10} response by the test drug was expressed as a percentage and calculated as: ([I GABA EC_{10} + modulator/I GABA EC_{10}] x 100), where I = current. The concentration-effect data were fitted to a four-parameter logistic equation (GraphPad Software, San Diego, CA). Data represent the mean ± S.E.M. (n = 3).[#] Data from Hogenkamp et al. (2014) and Carter et al. (1997); AlloP = allopregnanolone; EC_{50} = the concentration of steroid producing half the maximum response of that compound. Max Mod = the maximum modulation of the GABA EC_{10} response produced by the test compound.



Compound	$GABA_A$ Receptor Subtype.									
	α1β1γ2		α1β2γ2		α2β1γ2		α2β2γ2		α4β3δ	
	EC_{50} ± SEM (μM)	Max Mod ± SEM (% control)	EC_{50} ± SEM (μM)	Max Mod ± SEM (% control)	EC_{50} ± SEM (μM)	Max Mod ± SEM (% control)	EC_{50} ± SEM (μM)	Max Mod ± SEM (% control)	EC_{50} ± SEM (μM)	Max Mod ± SEM (% control)
AlloP	0.09 ± .02	817 ± 62	0.11 ± 0.01	909 ± 68	0.27 ± 0.20	878 ± 211	0.11 ± 0.04	485 ± 257	0.54 ± .09	1401 ± 88
Ganaxolone	0.13 ± 0.04	1006 ± 241	0.79 ± 0.63	1151 ± 367	0.1 ± 0.06[#]	700 ± 50[#]	0.19 ± 0.07	646 ± 254	0.25 ± 0.06	1710 ± 534
UCI-50027	0.63 ± 0.05	472 ± 84	1.33 ± 0.25	587 ± 91	0.34 ± 0.18	277 ± 47	1.41 ± 0.47	595 ± 176	1.04 ± 0.45	919 ± 229
SAGE-217	0.08 ± 0.02	488 ± 33	0.32 ± 0.16	823 ± 353	0.18 ± 0.04	851 ± 93	0.38 ± 0.06	1355 ± 173	0.15 ± 0.03	783 ± 122
2–261	1.2 ± 0.5	24 ± 8	0.36 ± 0.05	1073 ± 194	0.95 ± 0.14	105 ± 33	0.16 ± 0.04	921 ± 47	0.45 ± 0.1	1771 ± 294
2–329	1.7 ± 0.2	843 ± 136	0.044 ± 0.003	947 ± 244	0.41 ± 0.07	1055 ± 322	0.018 ± 0.008	559 ± 151	0.14 ± 0.01	2775 ± 99

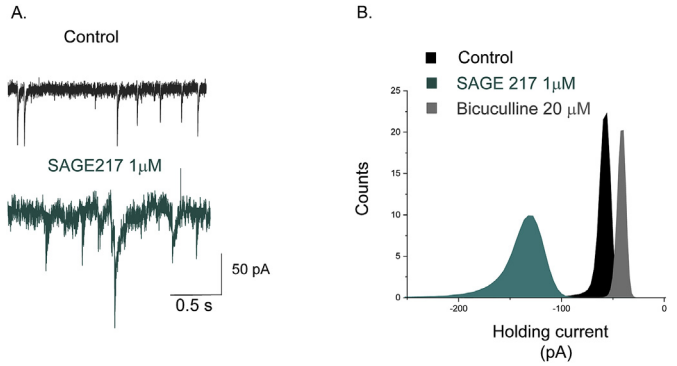


Fig. 1. *SAGE-217 greatly enhances both phasic and tonic inhibition $GABA_AR$ in mouse thalamic VB neurons*. **A)** $GABA_AR$ mIPSCs recorded at −70 mV from a representative VB neuron of a neonatal (P21) mouse horizontal slice before (Control, black trace) and following 10 min of SAGE 217 (1 μM, green trace). The mIPSCs appear as downward deflections from the baseline. Note the prolongation of the mIPSC decay by SAGE 217 and the simultaneous increase of the baseline noise. **B)** An all-points histogram of the holding current from the same recording depicted in A) under control conditions (black), following SAGE 217 (1 μM, green) and co-application of bicuculline (20 μM, grey). Note the large inward shift in the holding current after SAGE 217 (1 μM) and the reversal beyond the control holding current following bicuculline (20 μM), revealing a clear $GABA_AR$ tonic conductance. See Brown et al., 2015 for recording conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

are innervated by GABA-ergic nucleus reticularis (nRT) neurons. Murine VB neurons express synaptic (α1β2γ2) and extrasynaptic (α4β2δ) $GABA_ARs$, that mediate fast phasic and tonic inhibition respectively (Peden et al., 2008; Herd et al., 2013). During drowsiness and non-rapid eye movement (NREM) sleep these presynaptic nRT neurons fire with high frequency bursts, consequently releasing substantial amounts of GABA onto VB neurons, which in addition to engaging synaptic $GABA_ARs$, spills over from the synapse to activate peri- and extrasynaptic $GABA_ARs$. Under voltage-clamp this spillover GABA results in a greatly prolonged IPSC. The slow component of the IPSC is absent in $\alpha4^{-/-}$ mice and is prolonged by DS2, a δ-$GABA_AR$-selective PAM (Herd et al., 2013), confirming it to be mediated by activation of extrasynaptic $GABA_ARs$ (α4β2δ). For VB neurons allopregnanolone and related $GABA_AR$-active steroids greatly prolong fast phasic inhibition and increase the tonic current (Brown et al., 2015) – Fig. 1. Although the effects of neurosteroids on this spillover component of the IPSC remains to be determined, it is probable that allopregnanolone, in common with DS2 and etomidate (an intravenous general anesthetic), will greatly prolong such events (Herd et al., 2013, 2014). By contrast, benzodiazepines would be inert in this respect, causing only a relatively modest prolongation *via* the synaptic (α1β2γ2) component of the IPSC (Peden et al., 2008). Therefore, in some neurons the neurosteroid effect upon neural inhibition may be considerably greater than that of benzodiazepines (Weir et al., 2017).

A recent report highlights a further important, but indirect action of neurosteroids upon tonic inhibition (Parakala et al., 2019). In addition to binding to $GABA_ARs$, certain steroids, including allopregnanolone and progesterone activate a family of progesterone G-protein coupled receptors (p-GPCRs) located on the membrane surface (Pang et al., 2013; Schumacher et al., 2014; Parakala et al., 2019). The steroid ORG OD 02-0 (ORG) selectively activates these p-GPCRs, with no effect upon nuclear progesterone receptors and importantly, in contrast to allopregnanolone, has no direct interaction with $GABA_ARs$ (Parakala et al., 2019). Activation of p-GPCRs by ORG has no immediate effect upon phasic, or tonic inhibition of dentate granule neurons. However, prolonged p-GPCR activation by this steroid produced a delayed, selective and sustained increase of the tonic current, due to an increased cell surface expression of extrasynaptic $GABA_ARs$, with no effect on phasic inhibition (Parakala et al., 2019). This metabotropic effect of ORG and allopregnanolone on $GABA_AR$ trafficking resulted from phosphorylation of particular serine residues of the $GABA_AR$ β3 subunit *via* an involvement of PKA and PKC (Parakala et al., 2019). Importantly, although the neuroactive steroid ganaxolone acutely increased the tonic current of hippocampal DGGNs, in contrast to allopregnanolone it did not produce a sustained enhancement of this conductance (Modgil et al., 2017).

In summary, allopregnanolone interacts directly with $GABA_ARs$ to acutely prolong phasic and enhance tonic inhibition, but may exert an additional, metabotropic effect to increase tonic inhibition on a slower time scale, by selectively influencing extrasynaptic $GABA_AR$ trafficking. This form of sustained $GABA_AR$ plasticity may be important in understanding the therapeutic and physiological effects of allopregnanolone. In particular, it may underpin behavioural effects of allopregnanolone that remain following elimination of the steroid from the CNS. As highlighted by the differential profile of ganaxolone (Modgil et al., 2017), for future drug discovery programs, consideration of the activity of synthetic steroids upon the family of p-GPCRs may be warranted, in addition to the current focus on their direct interaction with the $GABA_AR$. In certain neurons, including DGGNs, the extrasynaptic δ-$GABA_ARs$ incorporate the β2 subunit (*e.g.* α4β2δ) – (Peden et al., 2008; Herd et al., 2008). It will be of interest to explore if this metabotropic effect extends to such receptors (see Modgil et al., 2017).

We will now consider attempts to develop neuroactive steroids as novel therapeutics, focussing primarily on indications where the $GABA_AR$ is the likely site of action and where possible clinical findings are available for evaluation.

3. Developing clinical applications of neuroactive steroids

3.1. General anesthetics

The initial clinical use of neuroactive steroids was as general anaesthetics. Following the discovery that progesterone exhibits both sedative and anticonvulsant efficacy in preclinical models (Selye, 1941), it was tested in humans intravenously (iv.) at 500 mg, a dose that produced an anesthetic effect (Merryman et al., 1954). This observation provided the impetus to examine the basis for this effect of progesterone and whether it had applications in developing new medicines. Based on pioneering work by Syntex on hypnotic steroids (Gyermek et al., 1968), in the 1970s Glaxo developed "Althesin" a combination of the steroids alphaxolone and alfadalone (Child et al., 1971). These steroids are analogues of allopregnanolone, the former has an 11-carbonyl substitution and the latter an 11-carbonyl, 21-hydroxyl substitution. As described (Introduction), alphaxalone is an efficacious $GABA_AR$ PAM. Clinically, Althesin was tested as a potential iv. induction agent undergoing clinical trials in the United States in the 1970s, but did not receive FDA approval for this application, although it was approved by regulatory agencies in Europe.

Althesin was formulated in a surfactant polyethylated castor oil (Cremophor). The attractive properties of Althesin, that distinguished it from other induction agents, were a lack of adverse cardiovascular, or respiratory effects, coupled with a limited post-recovery hangover due to a short half-life (Towler et al., 1982). However, Althesin caused anaphylaxis, resulting in withdrawal from human use, although it is used in veterinary practice (Barletta, 2019). The anaphylaxis was caused by histamine release induced by Cremophor and not the active components of Althesin (Johnson et al., 2011). An additional adverse effect of Althesin was hyperexcitability observed during induction and seizure activity during recovery (Towler et al., 1982). Minaxolone, a 2β-ethoxy, 11α-dimethylamino substituted analogue of allopregnanolone was also developed, but never marketed (Kharasch and Hollmann, 2015). Organon improved upon the poor solubility by creating a series of water-soluble neuroactive steroids with anesthetic properties, which were highly efficacious $GABA_AR$ PAMs (Hill-Venning et al., 1996;

Sneyd et al., 1997). These steroids included two candidates (Org-20599 and Org-21465) based on morpholinyl substitutions at the 2β-position on the steroid A ring and were advanced into clinical trials. In common with Althesin, their excitatory actions led to discontinuation of the trials (Gasior et al., 1999). Interestingly, a reformulated version of alphaxalone (Phaxan), which uses a cyclodextrin as a carrier, is currently in clinical development by Drawbridge Pharmaceuticals. This reformulation potentially eliminates the toxic effects of the original Cremophor formulation, but whether post-recovery excitability can be mitigated remains to be established.

The discovery of the GABA-modulatory effects of allopregnanolone encouraged exploring additional medical applications, common to established $GABA_AR$ modulators *e.g.* benzodiazepines.

3.2. Sedatives

Satisfactory sleep is crucial for physical and mental health (Krause et al., 2017). Various neuropsychiatric disorders including depression and schizophrenia are accompanied by disrupted sleep architecture. Benzodiazepines and zolpidem are widely prescribed for sleep disorders, but their side effects, including tolerance and withdrawal, make them far from ideal (Wafford and Ebert, 2008). Investigations of sedative applications of the allopregnanolone-related steroids have been sporadic, despite sedation being their most apparent behavioural effect. Clinical trials of Althesin and alphaxalone revealed sedative effects (Ramsay et al., 1974; Stewart et al., 1983). Neuroactive steroids have desirable attributes at sedative doses including a short duration of action, no hangover, no effects on sleep architecture, limited effects on cognition and an absence of cardiovascular and respiratory depression (Towler et al., 1982).

Although the neuroactive steroids were described as “barbiturate like” (Harrison and Simmonds, 1984; Majewska et al., 1986) they were subsequently shown to act upon a distinct site on the $GABA_AR$ (Introduction). This finding opened a path to patent (US patent No. 5,120,723) the use this novel site on the $GABA_AR$, by Gee and colleagues for the treatment of CNS disorders amenable to modulation by neuroactive steroids. This patent and others led to the development of synthetic neuroactive steroids related to allopregnanolone as drug candidates by CoCensys (Belelli et al., 1990; McNeil et al., 1992; Gee et al., 1995). They developed several orally bioavailable steroids for evaluation as sedative-hypnotics (Edgar et al., 1997; Vanover et al., 2001). In particular, CCD-3693, was more efficacious than benzodiazepines in promoting NREM sleep. In rats CCD-3693 did not interfere with rapid eye movement (REM) sleep and was more selective in reducing electroencephalogram (EEG) wakefulness, with less impairment of locomotor activity during waking than triazolam, or zolpidem (Edgar et al., 1997). In contrast to the benzodiazepines, neuroactive steroids do not produce a distinct “rebound” wakefulness following their NREM sleep-promoting effect. CCD-3693 was progressed into clinical development by CoCensys in collaboration with Searle in the 1990s, where it demonstrated similar properties on human sleep. Although CCD-3693 had a superior pharmacodynamic (PD) profile to that of benzodiazepines, it was not developed then because of pharmacoeconomic considerations.

Regarding mechanism, the sedative effects of the benzodiazepines are primarily mediated by α1βγ2 $GABA_ARs$ (Wafford and Ebert, 2008; Rudolph and Knoflach, 2011) and neuroactive steroids are effective PAMs of such receptors (Table 1; Fig. 1). However, the sleep times induced by allopregnanolone and alphaxalone are reduced in $\delta^{-/-}$ subunit mice, implicating extrasynaptic δ-$GABA_ARs$ (Mihalek et al., 1999). Similarly, the sedative effects of gaboxadol, a δ-$GABA_AR$-selective agonist (Belelli et al., 2005) are impaired in $\delta^{-/-}$ mice (Boehm et al., 2006; Wafford and Ebert, 2008; Herd et al., 2009). Although these studies identify a credible target for developing steroid based sedatives, their poor oral bioavailability remains a major impediment.

3.3. Anticonvulsant

The demonstration that a single iv. infusion of Althesin suppressed seizures in 7 of 9 patients with status epilepticus (SE) provided the first clinical evidence that steroids related to allopregnanolone have anticonvulsant activity (Munari et al., 1979). Clearly a logical and obvious indication for allopregnanolone was epilepsy. In support, allopregnanolone exhibits anticonvulsant efficacy in a number of preclinical models of seizures (Belelli et al., 1989; Luntz-Leybman et al., 1990; Kokate et al., 1994, 1996; Devaud et al., 1995; Kaminski et al., 2004; Reddy and Rogawski, 2012; Wu and Burnham, 2018). However, neither Althesin, nor allopregnanolone, were viable as orally active anticonvulsants, having plasma half-lives of < 1 h, limited solubility and oral bioavailability (Irwin et al., 2015). Side-stepping this impediment, a recent study described the successful treatment of two patients suffering from super-refractory SE following a two day continuous infusion with allopregnanolone (Vaitkevicius et al., 2017). Recently Sage Therapeutics, Inc. (Sage) took an *i.v.* formulation of allopregnanolone (brexanolone) into clinical testing for the treatment of drug refractory SE. The preparation, designated SAGE-547, showed promise in phase II trials, but failed to meet clinical endpoints in phase III (Rosenthal et al., 2017; Cox, 2017). Why SAGE-547 failed in phase III is not known. Perhaps the heterogeneous aetiology of SE undermines a response to the drug alone, or poor solubility prevented an optimal concentration from being achieved in the formulation used. Alternatively, a sustained allopregnanolone infusion may cause rapid PD tolerance (Turkmen et al., 2011). The clinical efficacy of allopregnanolone in large scale trials remains to be established in epilepsy, despite activity in preclinical models, or the anecdotal evidence of clinical activity of Althesin and other neuroactive steroids (Munari et al., 1979).

Ganaxolone, a synthetic analogue of allopregnanolone, was synthesised by CoCensys in the early 1990s to improve oral bioavailability, prevent *in vivo* conversion to progesterone and to increase the biological half-life (Carter et al., 1997). The introduction of a 3β-methyl group in ganaxolone significantly improved half-life, while retaining the $GABA_AR$ efficacy of allopregnanolone (Carter et al., 1997) –Table 1. This steroid was advanced by CoCensys into clinical trials in 1991 and showed promise as a potential antiepileptic (Reddy and Estes, 2016; Sperling et al., 2017). Ganaxolone was redirected for the treatment of acute migraine based on an uncertain rationale, but failed to demonstrate efficacy in phase II trials. Despite this costly detour, ganaxolone is currently under clinical development by Marinus Pharmaceuticals for treatment of various indications directed at seizure disorders, including cyclin-dependent kinase-like 5 deficiency disorder, protocadherin 19-related epilepsy and refractory SE (NCT03572933, NCT03865732, NCT03350035).

Recently, a series of novel (WO 2013/019711 A2) allopregnanolone-related steroids were designed to improve the aqueous solubility, bioavailability and side-effect profile of established neuroactive steroids and tested for antiepileptic activity (Hogenkamp et al., 2014). These 17β-heteroaryl steroids are represented by UCI-50027, the prototype in this series, 3-[3α-hydroxy-3β-methyl-5α-androstan-17β-yl]-5-(hydroxymethyl)isoxazole. UCI-50027 is an orally active anticonvulsant and anxiolytic, which overcomes the general dosing limitations of other neuroactive steroids. UCI-50027 has an improved preclinical pharmacokinetic (PK) and side-effect profile, which makes it more potent and tolerable as an anxiolytic and anticonvulsant than ganaxolone. For example, UCI-50027 has a greater anticonvulsant/anxiolytic therapeutic index than ganaxolone in rodent seizure models. Ganaxolone and UCI-50027 produce a robust enhancement of submaximal GABA-evoked currents in oocytes expressing human recombinant α1β2γ2L and α4β3δ $GABA_ARs$ (Table 1). Both compounds are potent modulators of α2β1γ2L $GABA_ARs$, but whereas ganaxolone greatly enhanced the response to GABA irrespective of the β subunit isoform, UCI-50027 produced only a relatively modest maximum modulation of α2β1γ2L, *c.f.* α2β2γ2L $GABA_ARs$ (Table 1). The differential influence of the β

subunit appeared selective for α2, but not the α1 subunit-containing $GABA_AR$ (Table 1). This reduced activity at the β1-subunit subtype may diminish the propensity to cause sedative/ataxic side effects *in vivo* at therapeutically relevant doses (Gee et al., 2010; Hogenkamp et al., 2014), although note the sedative effects of etomidate are mediated by $GABA_ARs$ incorporating the β2-subunit (Reynolds et al., 2003).

Preclinical and clinical evidence suggests that allopregnanolone and related analogues should be useful in the treatment of various epilepsies, but to date none have received regulatory approval. Perhaps a more targeted approach, that requires a precise matching of the underlying pathophysiology of the various seizure disorders with the mechanism of action (MOA) of neurosteroids, may be a prerequisite for success. In that regard, numerous genetic mutations of $GABA_AR$ subunits, implying impairments to phasic and tonic inhibition, associate with particular epilepsies, including mutations of the δ-subunit (Macdonald et al., 2010). Some clinical anticonvulsants increase ambient levels of GABA, suggesting manipulations to influence tonic inhibition may be beneficial in certain conditions (Brickley and Mody, 2012). However, note that enhancement of tonic inhibition in models of absence epilepsy aggravates the condition by promoting thalamic burst firing (Cope et al., 2009; Errington et al., 2011).

3.4. Post-partum depression (PPD)

Blockade of neurosteroid production by inhibition of 5α-reductase with finasteride increases depression-like behaviors in experimental animal models (Walf et al., 2006; Beckley and Finn, 2007) and in some men treated for male pattern hair loss *i.e.* "finasteride syndrome" (Locci and Pinna, 2017; Melcangi et al., 2017). Allopregnanolone levels are negatively correlated with depression-like behaviors (Walf et al., 2006). Moreover, in humans antidepressant treatments increase allopregnanolone levels, which may be an integral component of their therapeutic effect (Romeo et al., 1998; Uzunova et al., 1998; Schule et al., 2007, 2011; Locci and Pinna, 2017). These findings are consistent with the documented role of the hypothalamus, pituitary, adrenal axis (HPA) dysfunction in the aetiology of depression (Lupien et al., 2009) and the dynamic regulation of neurosteroid levels in response to stressful experiences (Purdy et al., 1991; Tomaselli and Vallee, 2019) to influence HPA axis activity (Patchev et al., 1994; Gunn et al., 2015).

Mirroring changes in progesterone, allopregnanolone levels rise steadily through pregnancy until shortly before childbirth, when they begin to fall, with a steep decline occurring immediately after birth (Luisi et al., 2000; Gilbert Evans et al., 2005; Paoletti et al., 2006: Locci and Pinna, 2017). One hypothesis suggests enhanced allopregnanolone levels during pregnancy may reduce expression of extrasynaptic α4βδ $GABA_ARs$, which remain depressed among patients who develop PPD (Maguire and Mody, 2008; Licheri et al., 2015; Osborne et al., 2017). In support, homozygous ($\delta^{-/-}$) and heterozygous ($\delta^{+/-}$) mice, impaired in their ability to decrease and upregulate δ-$GABA_AR$ expression during pregnancy and postpartum respectively, developed depression-like and abnormal maternal behaviors, resulting in reduced pup survival (Maguire and Mody, 2008), although see Gunn et al. (2013). These symptoms were reversed in the heterozygous $\delta^{+/-}$ mouse by the δ-$GABA_AR$ selective agonist gaboxadol (Maguire and Mody, 2008). If a similar dysregulation of receptor dynamics postpartum occurs in PPD then depressive symptoms may be exacerbated by fluctuating allopregnanolone levels and reduced δ-$GABA_AR$ expression.

Evidence relating perinatal mood and anxiety symptoms with absolute levels of allopregnanolone has been equivocal, especially in larger population studies. Reduced allopregnanolone levels are inversely correlated with depression scores in women during late pregnancy (Hellgren et al., 2014), but not in the second trimester (Hellgren et al., 2017). Interestingly, in this study a polymorphism in a gene involved in allopregnanolone synthesis, aldo-keto reductase family 1, which results in reduced allopregnanolone levels, was associated with an increase in depression scores. In another study second trimester cortisol, progesterone, pregnanolone and allopregnanolone levels were correlated with negative emotional symptoms (Crowley et al., 2016). Additional investigations demonstrated altered levels of GABA and neurosteroids in association with PPD (Deligiannidis et al., 2016). In contrast, mean allopregnanolone levels at 6 weeks postpartum in over 1500 ethnically diverse women showed no difference between healthy postpartum women and those with PPD (Guintivano et al., 2018). Low second trimester allopregnanolone levels were reported to predict subsequent PPD in a study of 60 pregnant women with mood disorders, where each additional ng/ml of this steroid reduced the probability of developing PPD by 63% (Osborne et al., 2017). However, there was no correlation when based on third trimester levels, consistent with another small study (Deligiannidis et al., 2013). It is not evident why levels during the third trimester showed no association. Studies reporting an association not with concurrent, but with subsequent symptoms, suggest that interactions with other systems, or other disease modifying changes (*e.g.*, receptor plasticity, gene transcription/expression, or changes to rate-limiting enzymes - Agis-Balboa et al., 2014) and other pleiotropic events may contribute to the complex interactions between allopregnanolone and perinatal symptoms.

Regardless, recent treatment trials of the synthetic iv. formulation of allopregnanolone (brexanolone), in phase III trials on PPD resulted in a statistically significant difference in remission rates between placebo and two different doses of brexanolone (Meltzer-Brody et al., 2018). Although not new since CoCensys (U.S. patent No. 5,120,723, issued 1992) anticipated the use of allopregnanolone for PPD, Sage was awarded breakthrough status and FDA approval for brexanolone as the first drug approved specifically for PPD. The steady-state plasma levels of brexanolone based on the C_{max} were in the 150–300 nM range during treatment infusion of PPD, corresponding to half the human EC_{50} for enhancement of α4β3δ $GABA_AR$ function by allopregnanolone (Brown et al., 2002). This magnitude of $GABA_AR$ facilitation would presumably be near maximal if the brain concentration is ×3 that in plasma, as observed in rodents (Irwin et al., 2015). Sage has rediscovered CoCensys' neuroactive steroids with a follow-on compound to brexanolone, SAGE-217, a synthetic orally-active allopregnanolone analogue that is covered generically in CoCensys patents (U.S. 6,143,736 issued Nov. 7, 2000 and U.S. 6,277,838 issued Aug. 21, 2001). They reported positive results with the compound in their phase II study when tested at 30 mg *p.o.* (once a day for 14 days). In common with allopregnanolone, SAGE-217 is a potent PAM of recombinant receptors, representative of both synaptic (α1/2β1/2γ2) and extrasynaptic (α4β3δ) $GABA_ARs$ (Table 1) – Martinez Botella et al. (2017). In agreement with this profile, for mouse thalamic VB neurons we find SAGE-217 (1 μM) to greatly prolong the duration of miniature inhibitory postsynaptic currents (mIPSCs) mediated by synaptic (α1β2γ2) $GABA_ARs$ and to enhance the tonic current mediated by extrasynaptic (α4β2δ) $GABA_ARs$ – Fig. 1.

Marinus is testing ganaxolone as an orally bioavailable treatment for PPD (NCT03228394, NCT03460756) with preliminary findings reported in the trade press as not meeting primary endpoints at an oral (p.o) dose of 1175 mg (once per day for 28 days). Given the similar $GABA_AR$ profile of these neuroactive steroids (Table 1) why is one steroid clinically active and the other inert? For example are they distinguished by their ability to activate the metabotropic progesterone receptor, leading to differences of δ-$GABA_AR$ trafficking (Modgil et al., 2017; Parakala et al., 2019)? Alternatively, the answer may reside in analysis of their pharmacokinetics, or other properties, revealed when these results are reported in peer-reviewed literature.

The intriguing possibility that neuroactive steroids have wider applications in clinical depression beyond PPD is now under investigation (Luscher and Mohler, 2019). A recent phase II study reported oral SAGE-217 treatment for 14 days to be effective in reducing depressive symptoms in patients with moderate to severe major depressive disorder (Gunduz-Bruce et al., 2019). The main side effects included dizziness and somnolence/sedation, not surprising given the $GABA_AR$

profile of this steroid (Table 1, Fig. 1).

If the apparent therapeutic effect is due primarily to acute facilitation of extrasynaptic δ-$GABA_AR$ function then other agents with a similar activity profile, may be useful in the treatment of PPD and other depressive disorders. Note benzodiazepines, which have no activity at δ-$GABA_ARs$, are not considered clinically useful in the treatment of depression *per se* (Baldwin et al., 2013), whereas clinically available drugs such propofol and etomidate, which in common with allopregnanolone, acutely enhance both phasic and tonic inhibition may be of interest (Belelli et al., 2005; Jia et al., 2007; Herd et al., 2013; Brown et al., 2015). However, note compounds such as propofol are unlikely to activate p-GPCRs to elicit a sustained effect upon δ-$GABA_AR$-mediated tonic inhibition, distinguishing them from allopregnanolone (Abramian et al., 2014; Parakala et al., 2019).

Finally, studies investigating the impact of depressive disorders on not only levels of allopregnanolone, but on the steroid metabolome, may prove instructive and identify additional steroid targets (Sharp et al., 2018; Tomaselli and Vallee, 2019).

3.5. Analgesia

The thalamus is associated with the processing of nociceptive signals (Steriade and Deschenes, 1984; McCormick, 1992; Oliveras and Montagne-Clavel, 1994). Thalamic sensory relay neurons receive GABA-ergic projections from sensory nuclei and the thalamic reticular nucleus (Steriade and Deschenes, 1984; McCormick, 1992). Microinjection of the $GABA_AR$ antagonist picrotoxin into the rat thalamic VB complex results in "pain-like" behavior (Oliveras and Montagne-Clavel, 1994). As discussed (Section 2), electrophysiological and immunohistochemistry studies reveal mouse VB neurons to express neurosteroid-sensitive synaptic (α1β2γ2) and extrasynaptic (α4β2δ) $GABA_ARs$ (Peden et al., 2008; Herd et al., 2013; Brown et al., 2015 – Fig. 1). Allopregnanolone is active in numerous models of acute and pathological pain states, with evidence of a neuroprotective effect (Kavaliers and Wiebe, 1987; Svensson et al., 2013; Borowicz et al., 2011; Rey and Coirini, 2015). The antinociceptive effects of allopregnanolone and ganaxolone are impaired in the $\delta^{-/-}$ mice (Mihalek et al., 1999). The δ-$GABA_AR$ agonist gaboxadol is antinociceptive in rodents and humans (Reyes-Vazquez and Dafny, 1983; Grognet et al., 1983; Lindeburg et al., 1983; Kjaer and Nielsen, 1983), but analgesic efficacy is attenuated in the $\alpha4^{-/-}$ mouse (Chandra et al., 2006). Collectively, these studies implicate extrasynaptic α4βδ $GABA_ARs$ in mediating, or contributing to the antinociceptive effects of neuroactive steroids, possibly by enhancing thalamic burst firing (Cheong et al., 2008) and identify a putative target for the development of pain therapeutics (Whissell et al., 2015). Clinically, allopregnanolone and related analogues have not been evaluated for activity in pain states. The lack of reports on self-administration and tolerance to the analgesic effects of allopregnanolone related drugs, in contrast to opioids, makes them worthy of consideration as analgesics.

3.6. Alzheimer's disease (AD) and neurodegenerative disorders

The role of allopregnanolone and related steroids in AD remains to be established as to whether it has a causal contribution, or is an epiphenomenon of the disease. It may be important to distinguish between the action of allopregnanolone as a PAM of the $GABA_AR$ *versus* actions at other sites (*e.g.,* pregnane X receptor, membrane bound p-GPCR – Section 2). In preclinical AD models allopregnanolone promotes neurogenesis, restores cognitive function and reduces pathology (Wang et al., 2010; Irwin et al., 2014; Ratner et al., 2019). The levels of allopregnanolone are reduced in the prefrontal cortex of AD patients (Marx et al., 2006). Perhaps $GABA_AR$ actions may contribute to the protective effects of allopregnanolone on neurodegeneration in AD models, but full efficacy may require a broader scope of interactions. However, the nature and extent of this contribution is unclear and may be minimal since ganaxolone (up to 30 mg/kg at 4 h post-injury) and pregnanolone (20 mg/kg) showed little effect in a preclinical model of cerebral hematoma, as measured by neurological and histological outcomes up to 3 months post-hematoma (Lyden et al., 2000). Additionally, progesterone failed to meet primary criteria in phase III clinical trials for the treatment of moderate to severe traumatic brain injury, although the contribution of allopregnanolone and related steroids, is unknown (Wright et al., 2014; Skolnick et al., 2014). Nevertheless, *i.v.* allopregnanolone has been tested in traumatic brain injury with equivocal results (NCT01673828).

Given the short metabolic half-life and absence of any reported PK/PD relationship at the $GABA_ARs$ in various AD models of cognitive deficits, among the most compelling argument for its MOA is a pleiotropic effect of allopregnanolone and related steroids, perhaps including an action upon p-GPCRs to influence $GABA_AR$ expression (Parakala et al., 2019, Section 2). Allopregnanolone is currently in clinical trials for the treatment of mild cognitive impairment due to AD (NCT02221622). It was reported to be safe and well-tolerated in the 2–16 mg intramuscular (im.) range in a small blinded- and placebo-controlled trial of 24 patients (both male and female, age 55 and above), with mild cognitive impairment due to AD, or mild AD (Brinton et al., 2018). In this study the CogState battery scores showed high variability, with no significant differences in cognitive measures between groups, though scores trended towards improvement with treatment. Although anecdotal, MRI studies reported allopregnanolone (4 mg dose, intramuscular im.) to improve functional connectivity between the right parietal lobe and posterior cingulate cortex and to exhibit a sustained effect on hippocampal volume over the 3 weeks of treatment (Brinton et al., 2018). These encouraging preliminary results will require confirmation by larger trials to determine the validity of the drug target in AD. Interestingly, the acute administration of allopregnanolone and other $GABA_AR$-active steroids to healthy subjects inhibits learning and memory function in a manner similar to the benzodiazepines (Bruins Slot et al., 1999; Johansson et al., 2002).

3.7. Beyond neurosteroids: non-steroid compounds selectively targeting α4βδ $GABA_AR$ subtypes

The clinical development of ganaxolone and allopregnanolone as drugs has relied on the use of patented formulations, which is driven by their poor physicochemical properties *e.g.* high lipophilicity (logP), which influences the bioavailability of the steroid. This major shortcoming is due to the intrinsic requirement of the androstane, or pregnane steroid core structure for efficacy (Hogenkamp et al., 2007). Although care is warranted regarding the interpretation of mouse gene deletion studies, the impaired behavioural efficacy of neuroactive steroids for either $\delta^{-/-}$ or $\alpha4^{-/-}$ mice identify extrasynaptic α4βδ $GABA_ARs$ as a target of interest in exploring the therapeutic potential of neuroactive steroids. As discussed (Section 2), the discovery that certain neuroactive steroids additionally activate p-GPCRs (Pang et al., 2013), leading to increased cell surface expression of δ-$GABA_ARs$ (Parakala et al., 2019) may warrant determining the efficacy of novel steroids upon this target, in addition to profiling their effect upon $GABA_AR$ isoforms.

Alternatively, structural chemotypes with PAM activity at α4βδ $GABA_ARs$, but improved physicochemical properties can be evaluated for indications for which neurosteroids have potential and demonstrated therapeutic utility. Currently there are few non-steroidal, small molecules that are selective for δ-$GABA_AR$ subtypes. DS2 is a highly selective PAM for extrasynaptic δ-$GABA_ARs$, exhibiting no activity on synaptic $GABA_ARs$, but it has poor CNS penetration and was not dosed orally for pharmacological evaluation (Wafford et al., 2009; Jensen et al., 2013). Nevertheless, DS2 is reported to improve recovery in a mouse model of stroke by an anti-inflammatory mechanism (Neumann et al., 2019). AA29504 (Hoestgaard-Jensen et al., 2010), has a favorable ratio of brain to plasma levels, but was not dosed orally, nor is it a

pure PAM since it also has direct agonist activity (Vardya et al., 2012). Furthermore, as a carbamate, AA29504 may be too unstable to be absorbed through the gut after oral dosing. The selective δ-GABA$_A$R agonist gaboxadol (in clinical development by Ovid Pharmaceuticals for treatment of Angelman and Fragile X syndromes) may worthy of consideration (Brown et al., 2002). However, the activity of δ-GABA$_A$R PAMs are governed in part by physiological firing patterns (Section 2), whereas this is presumably less so for direct agonists such as gaboxadol (Herd et al., 2013; Weir et al., 2017).

The non-steroidal small molecules 2–261 and 2–329 (Table 1), act as GABA$_A$R PAMs *via* a binding site distinct from that of neurosteroids, or benzodiazepines (Gee et al., 2010). They compare favorably to the neurosteroids, including those currently in clinical development, in terms of potency, efficacy and selectivity for GABA$_A$R subtypes including the α4β3δ isoform (Table 1). They display unique pharmacological properties, which can be readily manipulated through their novel SAR to produce desirable side effect/safety profiles based on GABA$_A$R receptor subunit-selectivity. For example, the orally bioavailable prototype, 2–261, retains anxiolytic activity without ataxia, cognitive impairment, ethanol potentiation, rewarding effects (linked to addiction), tolerance, or withdrawal (Gee et al., 2010; Yoshimura et al., 2014). Furthermore, in rodents 2–261 has a neurosteroid-like profile in blocking electrical- and chemical-kindled seizures and organophosphate-induced SE, and exhibits activity in chronic pain (Reddy et al., 2018; Johnstone et al., 2019a, b). Thus, retention of therapeutically desirable effects of the GABA$_A$R-active steroids, but not their side effects, is clearly advantageous. The freedom to operate in the patent space provided by small molecules yields a more flexible approach to drug development, that builds upon the extensive body of knowledge accumulated during the last three decades on neuroactive steroids and GABA$_A$Rs, but avoids their poor physicochemical properties and the prior art of the neurosteroid patent minefield.

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Delia Belelli – Original writing, subsequent reviewing and editing.

Kelvin Gee - Original writing, subsequent reviewing and editing.

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Jeremy Lambert – Original writing, subsequent reviewing and editing.

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