RESEARCH NOTE

Mitochondrial dysfunction in diabetic neuropathy may be involved in the development of neuropathic pain via a reduction in neurosteroid synthesis [version 1; referees: 1 approved, 1 approved with reservations]

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Abstract

Background: Recent work in a model of diabetic neuropathy revealed that layer 2/3 cortical pyramidal neurones of the pain pathway exhibited reduced endogenous neurosteroid modulation of the $GABA_AR$ and exogenously applied neurosteroids had an exaggerated impact. It is postulated that this is related to reduced precursor synthesis, due to mitochondrial dysfunction in diabetic neuropathy. Benzodiazepines are also known to activate neurosteroidogenesis by binding to mitochondrial translocator protein (TSPO). This study explored the differential effect of diazepam on $GABA_AR$ modulation via neurosteroidogenesis in diabetic and wild type (WT) mice.
Methods: Whole-cell patch-clamp technique was used on slices of neural tissue. Electrophysiological recordings were obtained from layer 2/3 cortical pyramidal neurons of the pain pathway from mice with type-II diabetic neuropathy (*ob/ob*) and WT controls aged 60-80 days.
Results: There was a key difference in the response of the WT and *ob/ob* cortical neurons to simultaneous incubation with diazepam and flumazenil. In contrast, diazepam and the 5a-reductase inhibitor finasteride, individually or in combination, produced the same response in both strains.
Conclusions: The exaggerated effect of diazepam on GABAergic inhibitory tone in the *ob/ob*, despite the presence of the $GABA_AR$ benzodiazepine antagonist flumazenil is likely observed due to physiological upregulation of key neurosteroidogenic enzymes in response to the reduced pregnenolone synthesis by the mitochondria. By increasing pregnenolone via TSPO activation, it is possible to promote enhanced neurosteroidogenesis and increase GABAergic inhibitory tone via an alternate route. In diabetic neuropathic pain, mitochondrial dysfunction may play an important role. Enhancing the GABAergic neurosteroid tone could be of potential therapeutic benefit.

Open Peer Review

Referee Status: ? ✔

	Invited Referees 1	Invited Referees 2
version 1 published 18 Apr 2017	? report	✔ report

1 **Slobodan M. Todorovic** , University of Colorado Anschutz Medical Campus, USA

2 **Pascal Darbon** , Centre National de la Recherche Scientifique and University of Strasbourg, France

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Comments (0)

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Introduction

Diabetic neuropathy is a common cause of painful neuropathy, and treatment is often suboptimal because the underlying aetiology is poorly understood. Peripheral and central sensitisation are implicated in the development of neuropathic pain with neuroplasticity occurring at multiple levels of the pain pathway (Harvey & Dickenson, 2008). GABAergic neurones at all levels of the pain pathway have a vital role in the transmission of painful stimuli in the perception of pain itself (D'Mello & Dickenson, 2008). Endogenous and exogenous neurosteroids may act as potent positive allosteric modulators of $GABA_A$ receptors ($GABA_A$Rs) and consequently exhibit analgesic, anxiolytic, anticonvulsant, and sedative properties (D'Hulst *et al.*, 2009).

Within inhibitory synapses, the presynaptic fusion of a single vesicle releases the inhibitory neurotransmitter GABA to activate synaptic $GABA_A$Rs. Under voltage-clamp conditions this causes a miniature inhibitory postsynaptic current (mIPSC). Drugs that enhance $GABA_A$R function cause a prolongation of the mIPSC decay phase. Recent work in a model of type-II diabetic neuropathy (*ob/ob*) revealed that layer 2/3 cortical pyramidal neurones of the pain pathway exhibited reduced endogenous neurosteroid modulation of the $GABA_A$R, and exogenously applied neurosteroids had an exaggerated impact (Humble, 2016a). The mechanism responsible appeared unrelated to $GABA_A$R sensitivity, but instead was associated with a reduction of neurosteroid precursors, such as pregnenolone, which is metabolised sequentially to the active compound allopregnanolone (Figure 1) (Humble, 2013; Humble, 2016a). Pregnenolone is synthesised in the mitochondrion from its precursor cholesterol by the side chain cleavage enzyme P450 located in the inner mitochondrial membrane (Do Rego *et al.*, 2009; Schumacher *et al.*, 2012), and it is postulated that diabetic neuropathy may be associated with a reduction in mitochondrial activity. Cholesterol is translocated across the mitochondrial membrane by the 18 kDa translocator protein (TSPO) in a coordinated fashion with the steroidogenic acute regulatory (StAR) protein (Do Rego *et al.*, 2009; Gatliff & Campanella, 2016; Rupprecht *et al.*, 2010; Stocco *et al.*, 2017; Figure 1).

The present study explored the impact of the benzodiazepine diazepam, a positive allosteric modulator of the $GABA_A$R (D'Hulst *et al.*, 2009), on $GABA_A$R modulation via neurosteroidogenesis in diabetic and wild type (WT) mice. Benzodiazepines are also known to activate neurosteroidogenesis by binding to TSPO (Rupprecht *et al.*, 2010; Tokuda *et al.*, 2010).

Methods

The methods are identical to those published by the same author previously (Humble, 2016a), with the exception of the drugs diazepam and flumazenil, which were not used in the previous study. Diazepam and flumazenil were purchased (Tocris, Bristol UK) and prepared as concentrated stock solutions in dimethyl sulfoxide before being added to the artificial extracellular solution as per the previous study (Humble, 2016a).

Results

Prolonged exposure (2 hrs) of mature cortical neurones to diazepam (1μM) in the presence of flumazenil had an exaggerated effect on the cortical $GABA_A$R-mediated mIPSCs of *ob/ob* mice in comparison to WT mice

Whole-cell voltage-clamp recordings were made in L2/3 cortical neurones of WT and *ob/ob* mice after at least two hours of incubation with diazepam, flumazenil and finasteride. Diazepam alone had the same effect on both strains of mice. In the WT mice, flumazenil inhibited the effect of diazepam (τ_W: control = 4.0 ± 0.1 ms, n = 35; finasteride 50 μM = 4.2 ± 0.1 ms, n = 7; diazepam 1 μM = 5.9 ± 0.2 ms, n = 6; flumazenil 10 μM & diazepam 1 μM = 4.0 ± 0.2 ms, n = 7; flumazenil 10 μM, finasteride 50 μM & diazepam 1 μM = 4.0 ± 0.3 ms, n = 6; One-way ANOVA, P <0.05; *post hoc* Newman Keul's test revealed a difference only for diazepam 1 μM, P <0.05; Figure 2). By contrast, in the *ob/ob* mice, flumazenil only partially inhibited the effect of diazepam, and the persisting effect of diazepam in the presence of flumazenil in the *ob/ob* mice could be prevented by the presence of the 5α-reductase enzyme inhibitor finasteride (τ_W: *ob/ob* control = 3.5 ± 0.1 ms, n = 25; finasteride 50 μM = 3.7 ± 0.2 ms, n = 6; diazepam 1 μM = 5.7 ± 0.3 ms, n = 6; flumazenil 10 μM & diazepam 1 μM = 4.9 ± 0.3 ms, n = 6; flumazenil 10 μM, finasteride 50 μM & diazepam 1 μM = 3.7 ± 0.1 ms, n = 5; One-way ANOVA, P <0.05; *post hoc* Newman Keul's test revealed significant intergroup differences for the flumazenil groups, P <0.05; Figure 2).

Discussion

Layer 2/3 cortical neurones from mature type-II diabetic *ob/ob* are known to have a reduced endogenous pregnane-derived neurosteroid tone in comparison to strain matched WT controls (Humble, 2016a). The present data indicate that by promoting the uptake of pregnenolone's precursor cholesterol by the mitochondria, via TSPO, diazepam may rescue the reduced neurosteroid tone. The restored neurosteroid tone could re-establish $GABA_A$R-mediated neuro-inhibitory tone in cases of neuropathic hypersensitivity. With specific reference to these data, the key result is the difference in response of the WT and *ob/ob* to simultaneous incubation with diazepam and flumazenil. In contrast, diazepam and the 5α-reductase inhibitor finasteride individually or in combination produced the same response in both WT and *ob/ob*. This may be interpreted as follows: in the WT, the primary effect of diazepam incubation is direct allosteric modulation of the $GABA_A$R, with negligible contribution from neurosteroidogenesis via mitochondrial TSPO activation. In comparison, diazepam has an exaggerated effect on GABAergic inhibitory tone in the *ob/ob*, despite the presence of the $GABA_A$R benzodiazepine antagonist flumazenil. This effect is likely observed due to physiological upregulation of the key rate-limiting enzymes involved in neurosteroidogenesis in response to the reduced pregnenolone synthesis by the mitochondria (Figure 1; Humble, 2016a). Thus by increasing the availability of the neurosteroid precursor pregnenolone via TSPO activation, it is possible to promote enhanced neurosteroidogenesis and thereby increase GABAergic

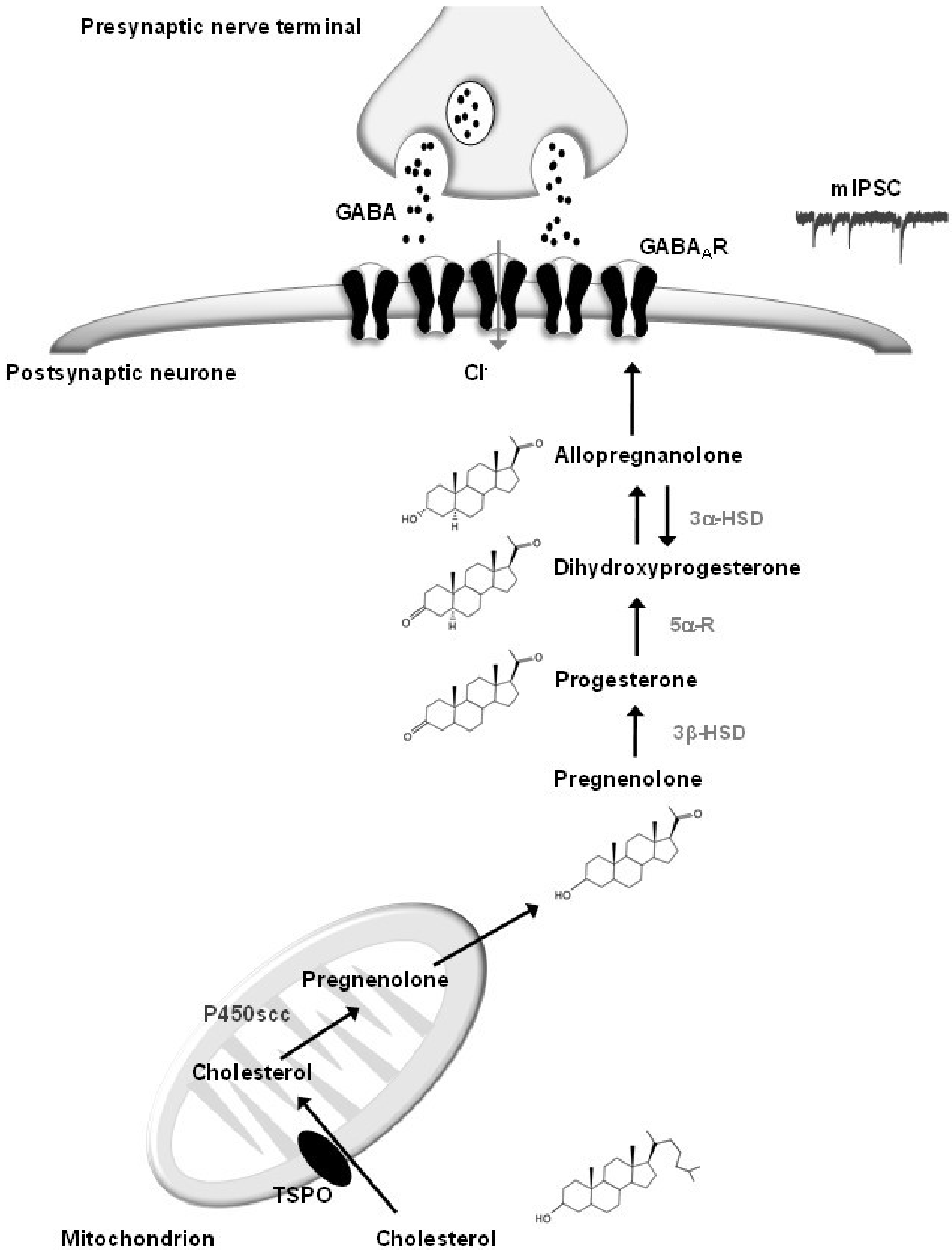

Figure 1. Modulation of the $GABA_AR$ by endogenous neurosteroids. Cholesterol is taken through the mitochondrial membrane by the translocator protein (TSPO) where it is converted to pregnenolone by the cytochrome P450 side chain cleavage enzyme. Pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD), which is in turn reduced to dihydroxyprogesterone by 5α-reductase (5α-R). Dihydroxyprogesterone is converted to allopregnanolone by 3α-hydroxysteroid dehydrogenase (3α-HSD). Postsynaptic $GABA_ARs$ are activated by GABA that has been released from vesicles in the presynaptic nerve terminal. GABA induces a conformational change of the $GABA_AR$, opening its central channel and thereby allowing the passage of chloride ions and the subsequent generation of miniature inhibitory postsynaptic currents (mIPSCs). The negative chloride ions induce hyperpolarisation of the neuronal membrane, which mediates neuronal inhibition. Neurosteroids, such as the active compound allopregnanolone, modulate $GABA_AR$ function and facilitate inhibition of the neuronal membrane. (Humble, 2013)

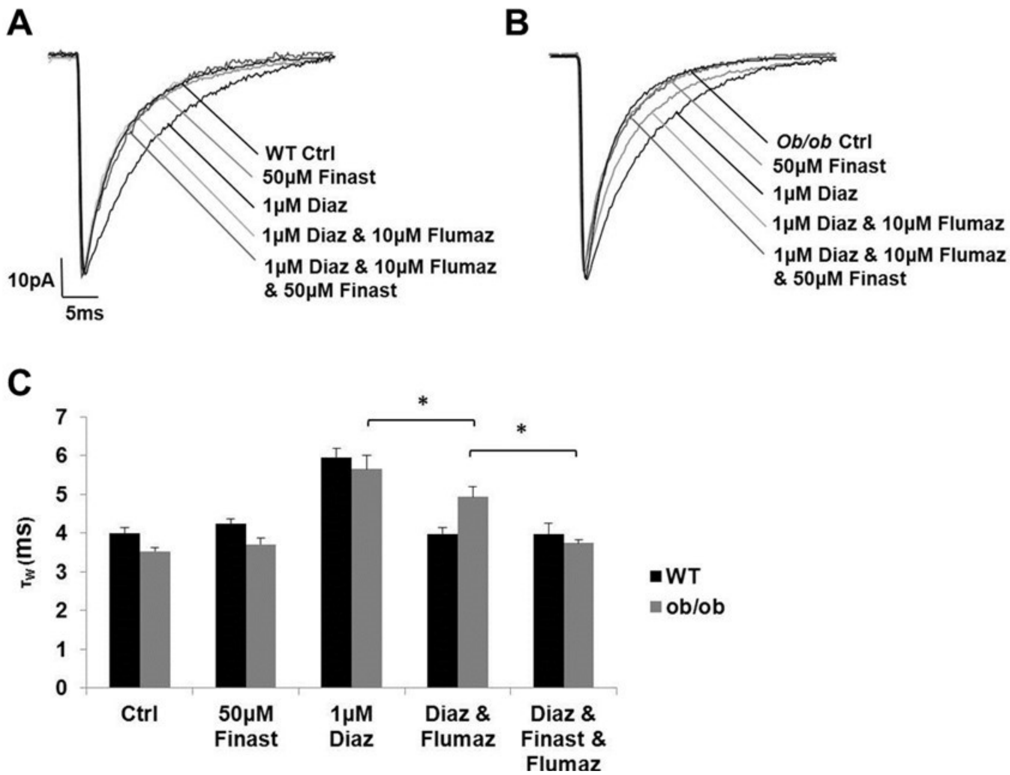

Figure 2. Prolonged exposure (2 hrs) of mature cortical neurones to diazepam (1μM) in the presence of flumazenil had an exaggerated effect on the cortical GABA$_A$R-mediated mIPSCs of *ob*/*ob* mice in comparison to WT mice. (**A**) Superimposed exemplar averaged GABA$_A$R-mediated miniature inhibitory postsynaptic currents (mIPSCs) acquired from a representative WT cortical neurone and from equivalent neurones after ~2 hours pre-incubation of the brain slice with diazepam (1μM), flumazenil (10 μM) and finasteride (50 μM). (**B**) Superimposed exemplar averaged GABA$_A$R-mediated mIPSCs acquired from a representative *ob/ob* cortical neurone and from equivalent neurones after ~2 hours pre-incubation of the brain slice with diazepam (1μM), flumazenil (10 μM) and finasteride (50 μM). (**C**) Histogram illustrating that flumazenil is able to prevent the effect of diazepam to prolong the duration of the GABA$_A$R-mediated mIPSC in WT cortical neurones, but only has a partial efficacy in *ob/ob* cortical neurones (τ_w in ms; one-way ANOVA, *P* >0.05; *Post hoc* Newman Keul's test). The persisting effect of diazepam in the presence of flumazenil in the *ob/ob* mice could be prevented by the presence of the 5α-reductase enzyme inhibitor finasteride (τ_w in ms; one-way ANOVA, *P* >0.05; *Post hoc* Newman Keul's test). Ctrl = control; Finast = finasteride; Diaz = diazepam; Flumaz = flumazenil.

inhibitory tone via an alternate route. Benzodiazepines modulate the GABA$_A$R by binding to the α-γ subunit interface (D'Hulst *et al.*, 2009), while neurosteroids bind the GABA$_A$R from a cavity within the α-subunit domain and modulate it directly via the α-β subunit interface (Hosie *et al.*, 2006). There have already been a number of other studies of ligands for the mitochondrial TSPO, as this is a promising target (Gatliff & Campanella, 2016; Giatti *et al.*, 2009; Papadopoulos & Lecanu, 2009; Rupprecht *et al.*, 2010; Zhang *et al.*, 2016). With reference to diabetic neuropathic pain and hypersensitivity, mitochondrial dysfunction may play an important role, and enhancing the GABAergic neurosteroid tone directly or indirectly could be of potential therapeutic benefit.

Data availability

Open Science Framework: Dataset of 'Neurosteroids are reduced in diabetic neuropathy and may be associated with the development of neuropathic pain', doi: 10.17605/osf.io/bk3tw (Humble, 2016b). Raw data for the present study can be found in Diazepam.zip.

Please refer to (Humble, 2016a) for details of standard software used for data analysis.

Author contributions

Dr Stephen Humble is responsible for this all work, including planning the experiments, performing the experiments and writing the paper. Prof Hales and Lambert, and Dr Belelli assisted Dr Humble with regards to the planning of some experiments. However, after discussion it was decided that their contributions merited being listed in the Acknowledgements section rather than as co-authors.

Competing interests

No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Supplementary material
Supplementary Tables 1 and 2: Analysed raw data.

Click here to access the data.

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Current Referee Status: ? ✓

Version 1

Referee Report 06 June 2017

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✓ **Pascal Darbon**
Institut des Neurosciences Cellulaires et Intégratives (INCI), UPR 3212 CNRS, Centre National de la Recherche Scientifique and University of Strasbourg, Strasbourg Cedex, France

The present article is a follow-up of Humble's previous paper in F1000Research (*Humble* SR. Neurosteroids are reduced in diabetic neuropathy and may be associated with the development of neuropathic pain [version 1; referees: 1 approved, 2 approved with reservations]. *F1000Research* 2016, **5** :1923 doi: 10.12688/f1000research.9034.1). The current article could be considered as an extended version providing further clarification on involved mechanisms. Therefore to fully understand the presented results, I recommend reading first the previous article.

However, in order to reach a broader leadership illustration and description could be improved. Figure 1 could be improved by highlighting the difference with Humble's 2016 paper; action sites of Diazepam, Flumazenil and Finasterid could be indicated. Likewise, results description focuses on difference between both mice strains without fully presenting individual results (Finast, Diaz, Flumaz…). This required a strong background in the field.

My last comment concerns the primary effect of diazepam on $GABA_A$R. The experiment consists in a 2 h incubation and it has been shown (*in vivo* in other brain structure, Zeitler *et al.* Eur J Neurosci, 2016) that Diazepam acts within 15-30 min then is relayed by neurosteroidogenesis. Therefore, it is difficult to conclude on the primary effect alone.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 14 Jun 2017

Stephen Humble, Imperial College London, UK

Dear Prof Darbon,

Thank you for your comments and for taking the time to review the paper. I agree that the illustration and description could be improved and I will do so as per your suggestions. I agree that the brief background description makes the research harder for a broad readership- this is as a result of the article's word limits.

Regarding the 2 hour incubation period for diazepam. This issue was considered at length during the experimental design stage and this is a relevant concern. It is of course true that *in vivo* diazepam acts within 15-30 minutes and that *in vivo* propofol and etomidate may act within seconds when given intravenously. However, previous work (Benkwitz *et al.,* 2007; Gredell *et al.,* 2004) has shown that in brain slice preparations the duration of onset may be much longer in lipophilic compounds such as these because the drug has to diffuse through the slice to reach the $GABA_A$ receptor on the neurone located within the slice. This contrasts to rapid drug delivery via an intact *in vivo* circulation, which may deliver the drug directly to the relevant neurone within seconds to minutes. Taking all this into account it was decided to use a 2 hour incubation period in order to maximise the impact of diazepam and highlight the difference between the diabetic neurones and the wild type. i.e. that diazepam in the presence of flumazenil (benzodiazepine receptor antagonist) had a greater response in the diabetic neurones, which appears to be mediated via upregulated neurosteroidogenesis and could be blocked by finasteride. Finally, I think that these results are not inconsistent with the Zeitler *et al.*, 2016 paper.

Yours sincerely

Stephen Humble

Competing Interests: No competing interests were disclosed.

Referee Report 30 May 2017

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?

Slobodan M. Todorovic
Department of Anesthesiology, School of Medicine, University of Colorado Anschutz Medical Campus, Aurora, CO, USA

My major concern with this study is that title and discussion are not relevant to the data presented. There is no justification to link this study to neuropathic pain or painful diabetic neuropathy. Perhaps more appropriate title would be:

"Mitochondrial dysfunction in an animal model of Type 2 diabetes may be involved reduction in neurosteroid synthesis "

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.