Rigor, Reproducibility, and In Vitro Cerebrospinal Fluid Assays: The Devil in the Details

Olivia A. Moody, Emory University
Sahil Talwar, University of Queensland
Meagan Jenkins, M. Jenkins Medical Communications
Amanda A Freeman, Emory University
Lynn Marie Trotti, Emory University
Paul Garcia, Emory University
Donald Bliwise, Emory University
Joseph W. Lynch, University of Queensland
Brad Cherson, Pavilion Compounding Pharmacy
Eric M. Hernandez, Noran Neurological Clinic

Only first 10 authors above; see publication for full author list.

Journal Title: Annals of Neurology
Volume: Volume 81, Number 6
Publisher: Wiley: 12 months | 2017-06-01, Pages 904-907
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1002/ana.24940
Permanent URL: https://pid.emory.edu/ark:/25593/sqbr9

Final published version: http://dx.doi.org/10.1002/ana.24940

Copyright information:
© American Neurological Association

Accessed April 5, 2022 9:31 AM EDT
Rigor, Reproducibility and *in vitro* CSF assays: The Devil in the Details

Olivia A. Moody, BA¹, Sahil Talwar, PhD², Meagan A. Jenkins, PhD³, Amanda A. Freeman, PhD⁴, Lynn Marie Trotti, MD, MSc⁵, Paul S. García, MD, PhD¹,⁶,⁷, Donald Bliwise, PhD⁵, Joseph W. Lynch²,⁸, Brad Cherson, RPh⁹, Eric M Hernandez, MD, PhD¹⁰, Neil Feldman, MD¹¹, Prabhjyot Saini, MSc⁵, David B. Rye, MD, PhD¹,⁵, and Andrew Jenkins, PhD¹,⁷,¹²

¹Program in Neuroscience, Graduate Division of Biological and Biomedical Sciences, Laney Graduate School, Emory University, Atlanta GA
²Queensland Brain Institute, The University of Queensland, Brisbane, QLD, Australia
³M. Jenkins Medical Communications, Decatur GA
⁴Center for the Study of Human Health, Emory College, Atlanta GA
⁵Department of Neurology and the Emory Program in Sleep, Emory University School of Medicine, Atlanta GA
⁶Anesthesiology and Research Divisions, Atlanta VA Medical Center, Atlanta, GA
⁷Department of Anesthesiology, Emory University School of Medicine, Atlanta GA
⁸School of Biomedical Sciences, The University of Queensland, Brisbane, QLD, Australia
⁹Pavilion Compounding Pharmacy, Atlanta, GA
¹⁰Noran Neurological Clinic, Minneapolis, MN
¹¹St. Petersburg Sleep Disorders Center, St. Petersburg, FL
¹²Department of Pharmacology, Emory University School of Medicine, Atlanta GA

Abstract
Divergent results and misinterpretation of non-significant findings remain problematic in science – especially in retrospective, hypothesis generating, translational research. When such divergence occurs, it is imperative that the cause of the divergence be established.

In their recent paper in *Annals of Neurology*, Dauvilliers et al challenged our earlier finding that cerebrospinal fluid (CSF) from some patients with unexplained excessive daytime sleepiness enhances the activation of GABA<sub>A</sub> receptors (GABA<sub>A</sub>-R). They present data from 15 subjects in which they were unable to find evidence of enhanced activation of GABA<sub>A</sub> receptors. Here we: 1) establish how flaws in Dauvilliers’ experimental design account for this difference; 2) present new data demonstrating the robustness and reproducibility of our methods and 3) summarize the clinical promise of GABA<sub>A</sub>-R antagonism in treating IH and related disorders.

### 1. Experimental Design

GABA<sub>A</sub>-R enhancement occurs when a positive allosteric modulator (PAM) increases agonist apparent affinity, resulting in increased chloride influx. This effect depends strongly on the effective concentration (EC) of the agonist that is used (i.e., what percentage of the maximal response the concentration of the agonist produces). To demonstrate this, we measured the effect of the CSF of a single subject with hypersomnolence on GABA<sub>A</sub>-Rs activated by a range of ECs of GABA (EC<sub>10-95</sub>, Figure 1A) using established methods. CSF enhanced GABA<sub>A</sub>-R activation at low ECs, but this effect diminished as EC increased (Figure 1B). In fact, at high concentrations of the agonist, which produce up to 95% of the maximal current flow through GABA<sub>A</sub>-R channels (EC<sub>95</sub>), a PAM can enhance desensitization, yielding smaller, even inhibitory, responses (Figure 1A and below). Thus, if the EC is too high, a powerful PAM will appear to have little or no effect. Put another way, a ceiling effect in Dauvilliers’ study obscures the effect of the CSF. For this reason, we always ensure our agonist concentration activates a response that is ~10% of the maximum (EC<sub>10</sub>) in a cell before testing a CSF sample.

### 2. Reproducibility

To emphasize the utility of our methods, 32 previously uncharacterized CSF samples were assessed by two independent electrophysiological methods at Emory University and the University of Queensland (Figure 1C). The correlation (r = 0.79) between the two replicates confirms that our methods are rigorous and our data are reproducible.

Dauvilliers et al. reported that they used an EC<sub>50</sub> GABA concentration in their CSF experiments. Below we demonstrate this to be erroneous, the actual EC is much higher. In their paper, the raw data shown in Dauvilliers’ concentration-response experiments demonstrate that low ECs activate plateaued responses whereas high ECs activate desensitizing responses that decrease rapidly despite the presence of GABA. Desensitization is strongly dependent on EC (Figure 1D). Using the relationship in Figure 1D, we can use the size of the decrease to get a better estimate of EC. Dauvilliers’ α1- and α2- raw data show a mean decrease of 11.6 ± 1.2%, n=15 and 15.9 ± 2.2%, n=6 respectively. Extrapolating from our concentration-desensitization relationships in Figure 1D, we find the EC in Dauvilliers’ CSF assays was on average closer to EC<sub>94</sub>, not EC<sub>50</sub> as reported. Under
these conditions, a CSF that enhanced EC\textsubscript{10} by 100% in our assays would only yield a 4% enhancement using Dauvilliers’ methodology and would likely require more than 250 replicates to reach statistical significance.

Additional methodological considerations undermine the reliability of Dauvilliers’ data. First, and as noted above, desensitization can reduce peak currents and be misinterpreted as inhibition. Both effects are magnified by PAMs (Figure 1A). Second, derivation of mean GABA\textsubscript{A}R enhancement values from single assessments of 2–4 individual \textit{Xenopus} oocytes exposure to 40 μl aliquots containing CSF, inadequately addresses sample dilution and other sources of variance expected for voltage-clamp electrophysiology (from the methods provided, it is not possible to say what the final CSF and GABA concentrations were in Dauvilliers’ study, since the dimensions of their bath and the volume of saline it contained is not stated). In contrast, we derive mean GABA\textsubscript{A}R enhancement values from 2–3 repeated uniform exposures of up to 7 individual HEK293 cells to CSF aliquots in a perfusion chamber with no aberrant time and dilution factors (\textit{i.e.}, 6–12 precise measurements). A key principle to replicating work done in another laboratory is that it be done in the same manner. Our ability to replicate GABA\textsubscript{A}R enhancement physiology in 32 novel CSF samples in a second laboratory suggests that Dauvilliers and colleague’s inability to do so is attributable to flaws in their experimental design.

3: Clinical Implications

Dauvilliers \textit{et al.} conclude that their findings: “do not favor the use of GABA\textsubscript{A}R antagonists (\textit{e.g.} flumazenil and clarithromycin) in patients with IH”. Basing therapeutic decisions on results of an \textit{in vitro} assay that is methodologically flawed, is a disservice to IH patients. Theirs is a challenging diagnosis of exclusion, for which approved treatments are lacking, and the psychosocial burden substantial.\textsuperscript{5} Clarithromycin might benefit sleepiness in IH by heightening neural excitability due to its being a negative allosteric modulator (NAM) of GABA\textsubscript{A}Rs,\textsuperscript{6} albeit, interference with soporific cytokines is another putative mechanism of action.\textsuperscript{7} Open label experience\textsuperscript{7} and a randomized, controlled trial\textsuperscript{8} of clarithromycin, used alone or with conventional wake-promoting drugs in narcolepsy type 2 and IH patients, demonstrate significant improvements in quality of life and sleepiness. Clarithromycin’s effectiveness in IH has been independently acknowledged\textsuperscript{9} and regularly observed in our diverse clinical practices (DBR, LMT, EMH, NF). Flumazenil is a competitive antagonist of benzodiazepines without intrinsic activity upon GABA signaling\textsuperscript{10–12}, so the most parsimonious explanation for its relieving sleepiness is via reversal of endogenous PAMs of GABA\textsubscript{A}Rs.\textsuperscript{13} Sustained, clinically-significant benefit was observed in \textasciitilde 40% of 153 treatment-refractory hypersonmnolet subjects with open-label use of compounded flumazenil\textsuperscript{13}

These studies, together with lack of consensus practice standards for treatment-refractory IH, have compelled our continued prescribing of compounded flumazenil. For the period of March 2013 through July 2016 (Fall 2014 start date for EMH and NF), prescriptions were filled by 344 unique patients from our three centers, with 45 (13%) refilled continuously for at least 6 months, and 18 (5%) uninterrupted for more than 2 years. These prescription-derived estimates of flumazenil’s effectiveness are probably overly conservative, given that

\textit{Ann Neurol}. Author manuscript; available in PMC 2018 June 01.
accessibility is limited by cost and difficulty of sourcing. Nonetheless, a pharmacy accounting for nearly all US-based compounded flumazenil prescriptions (BC) counts 89 additional prescribing physicians through mid-August, 2016. This experience adds to a substantial, growing scientific and clinical precedent that endogenous ligands of GABA<sub>A</sub>R modulate complex behaviors and are clinically relevant targets for novel therapeutics. One example is the repurposing of a GABA<sub>A</sub>R NAM as the first agent specifically targeting IH (Balance Therapeutics’ BTD-001), with a Phase Ib multi-center, randomized, controlled trial currently underway (NCT-025112588).

Finally, the range of GABA potentiation shown by Dauvilliers et al. (Figures 4A and 5A) indicate that human CSF can modulate responses to GABA. As we have described above, flawed experimental design can account for the modest or inhibitory actions of CSF on GABA<sub>A</sub>R function. Thus, despite methodological shortcomings, Dauvilliers et al.’s findings can ultimately be viewed as consistent with the presence of endogenous ligands within the brain that can modulate GABA receptor function.<sup>14–18</sup> What remains to be defined is their molecular identity and physiological effects upon sleep, which GABA<sub>A</sub>Rs and components mediate these effects, and with which symptoms, syndromes, and diseases it most closely associates.

Acknowledgments

This work was supported by: NIH: GM008602 & NS007480 (OM), NS083748 (LMT) & NS089719 (DBR, AJ), Veteran’s Affairs BX001677 (PSG), the James S. McDonnell (PSG) and Mind Science (DBR) Foundations, the Australian National Health and Medical Research Council APP1058542 (JWL) and the Queensland Emory Development Alliance (AJ).

References


Figure 1.
A. Example trace of currents recorded from $\alpha_1\beta_2\gamma_2$ GABA$_A$Rs in response to 10–300$\mu$M GABA ± 50% CSF. Dotted lines and arrows mark the degree/direction of modulation. Notice that low ECs result in enhancement while high ECs result in desensitization and inhibition. Scale bar: 5sec, 1000pA. B. Average enhancement (%) measured from peak currents ($I$): $((I_{GABA+CSF} - I_{GABA})/(I_{GABA}))$*100 for each GABA concentration shown in Figure A. (In Figure 1A, notice how enhancement is nearly zero at 30$\mu$M and negative beyond that as peaks desensitize, making measurements of enhancement unreliable. C.
Enhancement comparison of 32 CSFs in 2 populations of receptors: enhancement of $\alpha_2\beta_2$ receptors determined using planar patch clamp electrophysiology at the University of Queensland (Lynch Lab) and enhancement of $\alpha_1\beta_2\gamma_2$ receptors determined using single electrode patch clamp electrophysiology at Emory University (Jenkins Lab). The line represents the line of identity. D. Average desensitization of peak currents from $\alpha_1\beta_2\gamma_2$ (●) and $\alpha_2\beta_2\gamma_2$ (◊) receptors, calculated as the difference of peak amplitude to the amplitude at the end of each GABA exposure. Linear regressions to calculate desensitization (d%) as a function of log[GABA] for each receptor were: $\alpha_1$: $d\% = 8.67 \times \log[\text{GABA}] - 11.53$ and $\alpha_2$: $d\% = 18.12 \times \log[\text{GABA}] - 16.68$. Effective concentrations for each GABA concentration could be back calculated using Hill=1.36 ($\alpha_1$) and 1.53 ($\alpha_2$) and EC$_{50}= 60\mu M$ ($\alpha_1$) and 8.8$\mu M$ ($\alpha_2$) and the Hill equation: $I/I_{\text{max}} = [\text{GABA}]^nH/([\text{GABA}]^nH + \text{EC}_{50}^nH)$. Trace inset of a 2 sec exposure of saturating (300$\mu M$) GABA to $\alpha_2\beta_2\gamma_2$ receptors with dotted lines and arrow indicating the degree of desensitization. Scale bar: 1sec, 1000pA. n=24 cells ($\alpha_1$), n=35 cells ($\alpha_2$). Where not shown, the error bars are smaller than the symbol.