Rigor, Reproducibility and *in vitro* CSF assays: the Devil in the Details

Olivia A. Moody BA[1], Sahil Talwar PhD[2], Meagan A. Jenkins PhD[3], Amanda A. Freeman PhD[4], Lynn Marie Trotti MD, MSc[5], Paul S. García MD, PhD[1,6,7], Donald Bliwise PhD[5], Joseph W. Lynch[2,8], David B. Rye MD, PhD[1,5], Andrew Jenkins PhD[1,6,9]

1. Program in Neuroscience, Graduate Division of Biological and Biomedical Sciences, Laney Graduate School, Emory University, Atlanta GA
2. Queensland Brain Institute, The University of Queensland, Brisbane, QLD, Australia
3. M.Jenkins Medical Communications, Decatur GA
4. Center for the Study of Human Health, Emory College, Atlanta GA
5. Department of Neurology and Sleep Center, Emory University School of Medicine, Atlanta GA
6. Anesthesiology and Research Divisions, Atlanta VA Medical Center, Atlanta, GA
7. Department of Anesthesiology, Emory University School of Medicine, Atlanta GA
8. School of Biomedical Sciences, The University of Queensland, Brisbane, QLD, Australia.
9. Department of Pharmacology, Emory University School of Medicine, Atlanta GA.

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Address correspondence to:
Andrew Jenkins PhD, Associate Professor,
Departments of Anesthesiology and Pharmacology
1510 Clifton Rd NE #5013
Atlanta GA 30322

Phone: 404 727 3910
Fax: 404 727 0365
Email: ajenki2@emory.edu

Dauvilliers *et al*[1] negative study has challenged our discovery that human cerebrospinal fluid (CSF) enhances the activation of $GABA_A$ receptors ($GABA_A$-R) [2]. A companion letter[3] and data in Figure 1 demonstrate flaws in Dauvilliers' experimental design and underscore that our data are robust and reproducible, essential prerequisites for guiding successful clinical trials[4].

$GABA_A$-R enhancement occurs when a positive allosteric modulator (PAM) increases agonist apparent affinity. This effect depends strongly on the effective agonist concentration (EC) used. To demonstrate this, we measured the effect of a single subject's CSF on $GABA_A$-Rs activated by a range of ECs (EC_{10-95}, Figure 1A) using established methods[2]. CSF enhanced $GABA_A$-Rs at low ECs and this effect disappeared as EC increased (Figure 1B). Thus, if the EC is too high, a powerful PAM will appear to have little or no effect. For this reason, we always ensure our agonist concentration activates a response that is 10% of the maximum in every cell before testing a CSF sample (EC_{10}). To emphasize the utility of our method, we determined enhancement for 32 additional subjects in 2 separate laboratories, one at Emory, the other at the University of Queensland[2,5] (Figure 1C). The correlation (r = 0.79) between the two replicates confirmed our rigorous methods generate reproducible data.

Dauvilliers *et al.* failed to account for cell-cell EC variability in their CSF assays, on average using an EC_{94}, not EC_{50} as reported. Under these conditions, a CSF that enhanced EC_{10} by 100% would only yield a 4% enhancement and would

require more than 50 replicates to be statistically significant. Dauvilliers' concentration-response data 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) therefore the size of the decrease can be used to estimate EC. Dauvilliers' α1- and α2-data show a mean decrease of 11.6 ± 1.2%, n=15 and 15.9 ± 2.2%, n=6 respectively. Therefore, extrapolating from our concentration-desensitization relationships in Figure 1D, we find the EC in Dauvilliers' CSF assays was on average an EC_{94} and thus, too high to be useful:

Finally, desensitization can reduce peak currents, an effect increased by PAMs (see Figure 1A), resulting in CSF-mediated inhibition. This effect further undermines the reliability of Dauvilliers' data and we conclude their study does not establish an absence of effect, but instead suffers from flawed experimental design.

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References

1. Dauvilliers Y, Evangelista E, Lopez R, Barateau L, Jaussent I, Cens T, Rousset M, Charnet P. Absence of γ-aminobutyric acid-a receptor potentiation in central hypersomnolence disorders. Ann Neurol. 2016;80(2):259-6

2. Rye DB, Bliwise DL, Parker K, Trotti LM, Saini P, Fairley J, Freeman A, Garcia PS, Owens MJ, Ritchie JC, Jenkins A. Modulation of vigilance in the primary hypersomnias by endogenous enhancement of $GABA_A$ receptors. Sci Transl Med. 2012;4(161):161ra151.

3. Rye DB et al., Companion letter, Ann Neurol XXX

4. Begley CG, Ioannidis JP. Reproducibility in science: improving the standard for basic and preclinical research. Circ Res. 2015;116(1):116-26

5. Balansa W, Islam R, Fontaine F, Piggott AM, Zhang H, Xiao X, Webb TI, Gilbert DF, Lynch JW, Capon RJ. Sesterterpene glycinyl-lactams: a new class of glycine receptor modulator from Australian marine sponges of the genus Psammocinia. Org Biomol Chem. 2013 11(28):4695-701

Figure 1

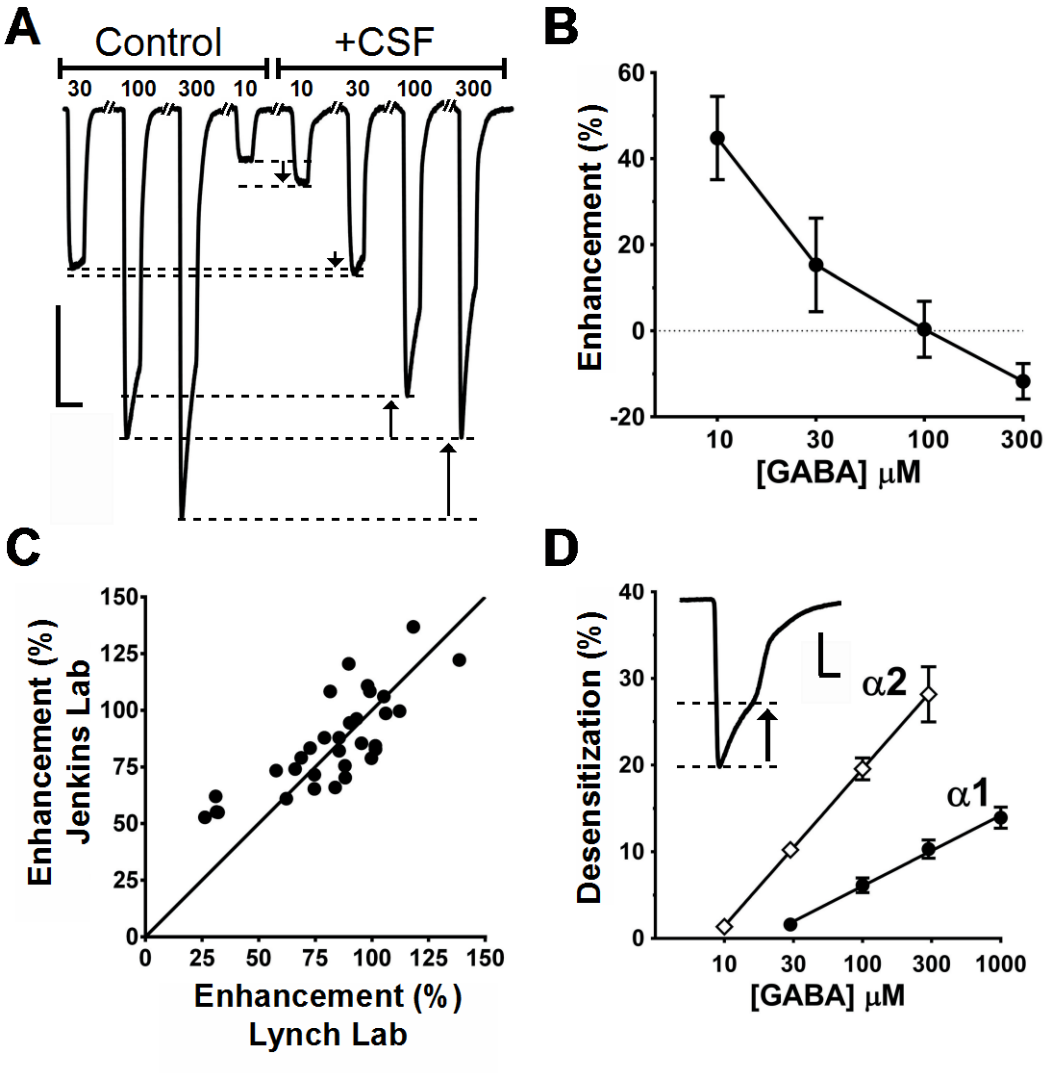
A
Control
+CSF
30 100 300 10 10 30 100 300
B
Enhancement (%)
60
40
20
0
-20
10 30 100 300
[GABA] μM
C
Enhancement (%)
Jenkins Lab
150
125
100
75
50
25
0
0 25 50 75 100 125 150
Enhancement (%)
Lynch Lab
D
Desensitization (%)
40
30
20
10
0
α2
α1
10 30 100 300 1000
[GABA] μM

Figure 1. A. Example trace of currents recorded from $\alpha_1\beta_2\gamma_2$ $GABA_A$Rs in response to 10- 300μM 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 30μM 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: α1: d%=8.67*log[GABA]-11.53 and α2: d%=18.12*log[GABA]-16.68. Effective concentrations for each GABA concentration could be back calculated using Hill=1.36 (α1) and 1.53 (α2) and EC_{50}= 60μM (α1) and 8.8μM (α2) and the Hill equation: $I/I_{max} = [GABA]^{nH}/([GABA]^{nH} + EC_{50}^{nH})$. Trace inset of a 2 sec exposure of saturating (300μM) 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

(α1), n=35 cells (α2). Where not shown, the error bars are smaller than the symbol.