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

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Abstract

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Author Contributions

DBR & AJ report prior financial support from Balance Therapeutics. DBR and AJ are co-inventors on United States Patent Application 20110028418 that describes the use of GABA_A receptor antagonists for the treatment of excessive sleepiness and sleep disorders associated with excessive sleepiness. Both have received royalty payments as part of the terms of sale of the related intellectual property to Balance Therapeutics. BC is co-owner of Pavilion Compounding Pharmacy, LLC, which is a for-profit entity that compounds flumazenil for patient use.

Conflict of Interests

Nothing to disclose for this work (see ICJME for interests not related to the current work): OAM, ST, MAJ, AFF, DB, LMT, EMH, NF, PS, PSG & JW. AJ, OAM, DBR, JW and LMT contributed to the conception and design of the study. OAM, ST, MAJ, AFF, AJ, PS, EMH, NF and BC were responsible for acquisition and analysis of the data. AJ, MAJ, OAM, LMT, PSG, JW, DBR and DB were responsible for drafting the text or preparing the figures.

Divergent results and misinterpretation of non-significant findings remain problematic in science – especially in retrospective, hypothesis generating, translational research.¹ 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*² challenged our earlier finding that cerebrospinal fluid (CSF) from some patients with unexplained excessive daytime sleepiness enhances the activation of GABA_A receptors (GABA_A-R)³. They present data from 15 subjects in which they were unable to find evidence of enhanced activation of GABA_A 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_A-R antagonism in treating IH and related disorders.

1. Experimental Design

GABA_A-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_A-Rs activated by a range of ECs of GABA (EC₁₀₋₉₅, Figure 1A) using established methods.³ CSF enhanced GABA_A-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_A-R channels (EC₉₅), 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 (EC₁₀) 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 University³ and the University of Queensland⁴ (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 EC₅₀ 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' $\alpha 1$ - and $\alpha 2$ - raw data show a mean decrease of $11.6 \pm 1.2\%$, $n=15$ and $15.9 \pm 2.2\%$, $n=6$ respectively. Extrapolating from our concentration-desensitization relationships in Figure 1D, we find the EC in Dauvilliers' CSF assays was on average closer to EC₉₄, not EC₅₀ as reported. Under

these conditions, a CSF that enhanced EC₁₀ 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_AR enhancement values from single assessments of 2–4 individual *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_AR 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 (*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_AR 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 *et al.* conclude that their findings: “do not favor the use of GABA_AR antagonists (e.g. flumazenil and clarithromycin) in patients with IH”. Basing therapeutic decisions on results of an *in vitro* assay that is methodologically flawed, is a disservice to IH patients. There is a challenging diagnosis of exclusion, for which approved treatments are lacking, and the psychosocial burden substantial.⁵ Clarithromycin might benefit sleepiness in IH by heightening neural excitability due to its being a negative allosteric modulator (NAM) of GABA_ARs,⁶ albeit, interference with soporific cytokines is another putative mechanism of action.⁷ Open label experience⁷ and a randomized, controlled trial⁸ 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 acknowledged⁹ 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^{10–12}, so the most parsimonious explanation for its relieving sleepiness is via reversal of endogenous PAMs of GABA_ARs.¹³ Sustained, clinically-significant benefit was observed in ~40% of 153 treatment-refractory hypersomnolent subjects with open-label use of compounded flumazenil.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

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_AR modulate complex behaviors and are clinically relevant targets for novel therapeutics. One example is the repurposing of a GABA_AR NAM as the first agent specifically targeting IH (Balance Therapeutics' BTD-001), with a Phase IIb 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_AR 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.^{14–18} What remains to be defined is their molecular identity and physiological effects upon sleep, which GABA_ARs and components mediate these effects, and with which symptoms, syndromes, and diseases it most closely associates.

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References

1. Altman DG, Bland JM. Absence of evidence is not evidence of absence. *BMJ*. 1995; 311(7003):485. [PubMed: 7647644]
2. Dauvilliers Y, Evangelista E, Lopez R, et al. Absence of gamma-aminobutyric acid-a receptor potentiation in central hypersomnolence disorders. *Annals of neurology*. 2016; 80:259–268. [PubMed: 27315195]
3. Rye DB, Bliwise DL, Parker K, et al. Modulation of vigilance in the primary hypersomnias by endogenous enhancement of GABAA receptors. *Sci Transl Med*. 2012; 4:161ra151.
4. Balansa W, Islam R, Fontaine F, et al. Sesterterpene glycyl-lactams: a new class of glycine receptor modulator from Australian marine sponges of the genus *Psammocinia*. *Org Biomol Chem*. 2013; 11:4695–4701. [PubMed: 23760172]
5. Billiard M, Sonka K. Idiopathic hypersomnia. *Sleep Med Rev*. 2016; 29:23–33. [PubMed: 26599679]
6. Bichler EK, Elder CC, Garcia PS. Clarithromycin increases neuronal excitability in CA3 pyramidal neurons through a reduction in GABA-ergic signaling. *Journal of neurophysiology*. 2016 jn 00134 02016.
7. Trotti LM, Saini P, Freeman AA, et al. *Journal of Psychopharmacology*. Vol. 28. Oxford, England: 2014. Improvement in daytime sleepiness with clarithromycin in patients with GABA-related hypersomnia: Clinical experience; p. 697-702.
8. Trotti LM, Saini P, Bliwise DL, Freeman AA, Jenkins A, Rye DB. Clarithromycin in gamma-aminobutyric acid-Related hypersomnolence: A randomized, crossover trial. *Annals of Neurology*. 2015; 78:454–465. [PubMed: 26094838]
9. Mignot EJM. *A Practical Guide to the Therapy of Narcolepsy and Hypersomnia Syndromes*. *Neurotherapeutics*. 2012; 9:739–752. [PubMed: 23065655]

10. File SE, Pellow S. Intrinsic actions of the benzodiazepine receptor antagonist Ro 15-1788. *Psychopharmacology (Berl)*. 1986; 88:1–11. [PubMed: 3003778]
11. Whitwam JG, Amrein R. Pharmacology of flumazenil. *Acta Anaesthesiol Scand Suppl*. 1995; 108:3–14. [PubMed: 8693922]
12. Neave N, Reid C, Scholey AB, et al. Dose-dependent effects of flumazenil on cognition, mood, and cardio-respiratory physiology in healthy volunteers. *British Dental Journal*. 2000; 189:668–674. [PubMed: 11191179]
13. Trotti LM, Saini P, Koola C, LaBarbera V, Bliwise DL, Rye DB. Flumazenil for the Treatment of Refractory Hypersomnolence: Clinical Experience with 153 Patients. *J Clin Sleep Med*. 2016; 12:1389–1394. [PubMed: 27568889]
14. Cravatt BF, Prospero-Garcia O, Siuzdak G, Gilula NB, Henriksen SJ, Boger DL, Lerner RA. Chemical characterization of a family of brain lipids that induce sleep. *Science*. 1995; 268(5216): 1506–9. [PubMed: 7770779]
15. Rothstein JD, Guidotti A, Tinuper P, Cortelli P, Avoni P, Plazzi G, Lugaresi E, Schoch P, Montagna P. Endogenous benzodiazepine receptor ligands in idiopathic recurring stupor. *Lancet*. 1992; 340(8826):1002–4. [PubMed: 1357403]
16. Guidotti A, Ferrero P, Costa E. On the brain endocoid for benzodiazepine recognition sites. *Prog Clin Biol Res*. 1985; 192:477–84. [PubMed: 3001768]
17. Alfonso J, Le Magueresse C, Zuccotti A, Khodosevich K, Monyer H. Diazepam binding inhibitor promotes progenitor proliferation in the postnatal SVZ by reducing GABA signaling. *Cell Stem Cell*. 2012; 10(1):76–87. [PubMed: 22226357]
18. Christian CA, Herbert AG, Holt RL, Peng K, Sherwood KD, Pangratz-Fuehrer S, Rudolph U, Huguenard JR. Endogenous positive allosteric modulation of GABA(A) receptors by diazepam binding inhibitor. *Neuron*. 2013; 78(6):1063–74. [PubMed: 23727119]

Figure 1.

A. Example trace of currents recorded from $\alpha_1\beta_2\gamma_2$ GABA_ARs in response to 10–300 μ M GABA $\pm$ 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): $(I_{\text{GABA+CSF}} - I_{\text{GABA}})/I_{\text{GABA}} \times 100$ for each GABA concentration shown in Figure A. (In Figure 1A, notice how enhancement is nearly zero at 30 μ 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: α_1 : $d\% = 8.67 * \log[\text{GABA}] - 11.53$ and α_2 : $d\% = 18.12 * \log[\text{GABA}] - 16.68$. Effective concentrations for each GABA concentration could be back calculated using Hill=1.36 (α_1) and 1.53 (α_2) and $EC_{50} = 60\mu\text{M}$ (α_1) and $8.8\mu\text{M}$ (α_2) and the Hill equation: $I/I_{\text{max}} = [\text{GABA}]^{nH} / ([\text{GABA}]^{nH} + EC_{50}^{nH})$. Trace inset of a 2 sec exposure of saturating ($300\mu\text{M}$) 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 (α_1), n=35 cells (α_2). Where not shown, the error bars are smaller than the symbol.