2. Stuth EA, Stucke AG, Brandes IF, Zuperku EJ: Anesthetic effects on synaptic transmission and gain control in respiratory control. Respir Physiol Neurobiol 2008; 164:151–9
3. Guyenet PG: The 2008 Carl Ludwig Lecture: Retrotrapezoid nucleus, CO_2 homeostasis, and breathing automaticity. J Appl Physiol (1985) 2008; 105:404–16
4. Nattie E, Li A: Muscimol dialysis in the retrotrapezoid nucleus region inhibits breathing in the awake rat. J Appl Physiol (1985) 2000; 89:153–62
5. Nattie E, Shi J, Li A: Bicuculline dialysis in the retrotrapezoid nucleus (RTN) region stimulates breathing in the awake rat. Respir Physiol 2001; 124:179–93
6. Gillis RA, Namath IJ, Easington C, Abrahams TP, Guidotti A, Quest JA, Hamosh P, Taveira da Silva AM: Drug interaction with gamma-aminobutyric acid/benzodiazepine receptors at the ventral surface of the medulla results in pronounced changes in cardiorespiratory activity. J Pharmacol Exp Ther 1989; 248:863–70
7. Flögel CM, Ward DS, Wada DR, Ritter JW: The effects of large-dose flumazenil on midazolam-induced ventilatory depression. Anesth Analg 1993; 77:1207–14
8. Gross JB, Weller RS, Conard P: Flumazenil antagonism of midazolam-induced ventilatory depression. Anesthesiology 1991; 75:179–85
9. Mora CT, Torjman M, White PF: Sedative and ventilatory effects of midazolam infusion: Effect of flumazenil reversal. Can J Anaesth 1995; 42:677–84
10. Chemali JJ, Van Dort CJ, Brown EN, Solt K: Active emergence from propofol general anesthesia is induced by methylphenidate. Anesthesiology 2012; 116:998–1005
11. Solt K, Cotten JF, Cimenser A, Wong KF, Chemali JJ, Brown EN: Methylphenidate actively induces emergence from general anesthesia. Anesthesiology 2011; 115:791–803
12. Taylor NE, Chemali JJ, Brown EN, Solt K: Activation of D1 dopamine receptors induces emergence from isoflurane general anesthesia. Anesthesiology 2013; 118:30–9

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In Reply:

We thank Dr. Raines for his interest in our work and thoughtful comments on the molecular pharmacology underlying our observed effects.

Our study[1] was primarily motivated by commonly encountered clinical scenarios, and our conclusions focused on network and *in vivo* actions of the combination of flumazenil in the setting of decreasing isoflurane concentration. While we are confident in concluding that flumazenil modulates emergence in rodents, there are insufficient data to completely describe the range of pharmacologic interactions between flumazenil and γ-aminobutyric acid (GABA) type A receptors ($GABA_A$Rs), including site-specific interactions. Our demonstration of "antagonistic activity" by flumazenil on $GABA_A$Rs in heterologous expression systems in the presence and absence of coapplied isoflurane mainly served to emphasize a GABA-mediated effect of flumazenil and isoflurane.

Based on our previous work[2,3] and that of several other successful laboratories[4–6] focused on GABA pharmacology, we are forced to constantly reevaluate the biophysical relationships of these compounds with the $GABA_A$R. We still struggle with a comprehensive understanding of moiety-specific interactions with regard to the effects of flumazenil on binding, gating, desensitization, and/or membrane regulation of the $GABA_A$R.

Our manuscript demonstrates that flumazenil robustly inhibits the enhancement of GABA-mediated current by isoflurane. However, in isolation, our work is insufficient to ascribe a precise mechanism of action, and we accept Dr. Raines's assertion that the antagonism may not be competitive. By the same token, however, from the dataset provided, it is not possible to confidently say that flumazenil is a noncompetitive antagonist. Nor is it possible to say at which site(s) flumazenil is acting. The intrinsic efficacy we demonstrate suggests that it is also a partial agonist. So, without a battery of new pharmacology experiments, we are in agreement that (1) the sites and mechanisms of flumazenil action are far from well understood and (2) the status of flumazenil as a simple benzodiazepine competitive antagonist should be called into question.

During the construction of the published version of this manuscript, the electroencephalogram and behavioral results were emphasized, and some of the pharmacologic descriptions were eliminated or simplified. Early versions of the manuscript described flumazenil as a "negative allosteric modulator of the $GABA_A$R at site(s) unknown." However, this wording is vague and imprecise. We decided it would be most appropriate to use similar wording to that in our human studies in this area.[3] We look forward to following the work of others in this area to improve the collective knowledge of $GABA_A$R pharmacology as applied to general anesthesia.

We also thank Drs. Petrenko and Baba for their interest in our work from a respiratory physiology perspective. In their letter, they raise the possibility that the influence of flumazenil on emergence from isoflurane anesthesia observed in our rodent model may not be entirely mediated by the neurophysiologic changes observed in cortical neurons but could be, in part, influenced by an effect of flumazenil on brainstem nuclei controlling respiration.

As evidence for influence of this alternative mechanism, they provide references to work in the *awake* rat that demonstrates respiratory changes when traditional GABA agonists[7] and antagonists[8] are applied by microdialysis to the retrotrapezoid nucleus in the ventral medulla. As discussed above, flumazenil does not affect the $GABA_A$R like the traditional agonists/antagonists, *i.e.*, muscimol and bicuculline. We are aware that GABA, in combination with adenosine, glutamate, and other neurotransmitters, is involved in regulating breathing in the retrotrapezoid nucleus, as well as other structures in the ventral respiratory group, and that a complex interplay exists between the ventral respiratory group and higher order structures (*e.g.*, pons, hypothalamus, and cortex).[9] For this reason, we carefully considered an influence of respiration on our results. Before initiation of our study, we performed a small (n = 6) pilot study on rats under near-identical conditions, and blood-gas measurements from these animals revealed

physiologically normal blood acidity (pH = 7.398) and slightly elevated partial pressure of carbon dioxide (48.6) at the end of the isoflurane anesthetic exposure. This can be sufficiently explained by the reduced respiratory rate of rodents under isoflurane anesthesia (1.2 to 2%). Our results are consistent with those of other investigators,[10] who reported that rodents at this concentration of isoflurane respire more slowly (60 to 100 breaths/min). The respirations are consistently observable as profound chest/abdominal excursions, and our anecdotal observation is that the rodents are breathing at normal (or slightly larger) tidal volumes while anesthetized with isoflurane. This assumption is supported by the near-normal blood gas measurements in our pilot studies. Upon administration of flumazenil, we did not observe any apparent differences in chest excursion. We carefully measured respiratory rate in our experiments, as tidal volume would have been very difficult to measure in our setup while maintaining the relevance to the clinical situation of emergence in the operating room. Furthermore, adding an additional invasive procedure such as blood gas measurements *via* intracardiac blood sampling would invalidate our behavioral measurements of emergence.

Drs. Petrenko and Baba also provide references to human studies involving reversal of midazolam with flumazenil. As we did not use midazolam in our study, we find the effects of flumazenil on midazolam-induced respiratory changes irrelevant to our findings.

We are very familiar with the work by Dr. Solt's research group on the pharmacology of active emergence[11–13] and applaud their elegant demonstration of immediate transitions to arousal during steady-state administration of low-dose anesthesia.[13] Our study aimed to more closely reproduce, in a reductive setting, the clinical conditions under which flumazenil may be used as a reversal agent. For this reason, we purposefully chose to characterize behavioral endpoints during the decrease of anesthetic concentration that occurs after cessation of general anesthesia. Although this approach cannot determine the actual anesthetic concentration at which the transition to arousal takes place, it more closely mirrors the clinical situation, where a variety of factors (*e.g.*, auditory stimulation, postsurgical pain, and individual heterogeneity in anesthetic sensitivity) influence the transition from unconsciousness to arousal. We agree that replicating our experiments in conditions of steady-state, low-dose isoflurane anesthesia could provide interesting results and will take that under consideration as we investigate the molecular mechanisms of flumazenil in greater detail.

Overall, we are pleased that Drs. Petrenko and Baba agree with the practical implications of our work. They suggest that flumazenil could be a valuable tool to facilitate recovery in clinical situations. Despite their concerns about the possible effects of flumazenil on respiration, we conclude that it is unlikely that the modulation of emergence by flumazenil is driven by changes in respiration, and that any minor effects of flumazenil on GABA signaling in the respiratory circuits were noncontributory in our study.

Competing Interests

The authors declare no competing interests.

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References

1. Safavynia SA, Keating G, Speigel I, Fidler JA, Kreuzer M, Rye DB, Jenkins A, García PS: Effects of γ-aminobutyric acid type A receptor modulation by flumazenil on emergence from general anesthesia. Anesthesiology 2016; 125:147–58
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 GABAA receptors. Sci Transl Med 2012; 4:161ra151
3. Richardson JE, Garcia PS, O'Toole KK, Derry JM, Bell SV, Jenkins A: A conserved tyrosine in the beta2 subunit M4 segment is a determinant of gamma-aminobutyric acid type A receptor sensitivity to propofol. Anesthesiology 2007; 107:412–8
4. Rüsch D, Forman SA: Classic benzodiazepines modulate the open-close equilibrium in alpha1beta2gamma2L gamma-aminobutyric acid type A receptors. Anesthesiology 2005; 102:783–92
5. Rüsch D, Zhong H, Forman SA: Gating allosterism at a single class of etomidate sites on alpha1beta2gamma2L GABA A receptors accounts for both direct activation and agonist modulation. J Biol Chem 2004; 279:20982–92
6. Scheller M, Forman SA: Coupled and uncoupled gating and desensitization effects by pore domain mutations in GABA(A) receptors. J Neurosci 2002; 22:8411–21
7. Nattie E, Li A: Muscimol dialysis in the retrotrapezoid nucleus region inhibits breathing in the awake rat. J Appl Physiol (1985) 2000; 89:153–62
8. Nattie E, Shi J, Li A: Bicuculline dialysis in the retrotrapezoid nucleus (RTN) region stimulates breathing in the awake rat. Respir Physiol 2001; 124:179–93
9. Bianchi AL, Gestreau C: The brainstem respiratory network: An overview of a half century of research. Respir Physiol Neurobiol 2009; 168:4–12
10. Dardai E, Heavner JE: Respiratory and cardiovascular effects of halothane, isoflurane and enflurane delivered *via* a Jackson-Rees breathing system in temperature controlled and uncontrolled rats. Methods Find Exp Clin Pharmacol 1987; 9:717–20
11. Chemali JJ, Van Dort CJ, Brown EN, Solt K: Active emergence from propofol general anesthesia is induced by methylphenidate. Anesthesiology 2012; 116:998–1005
12. Kenny JD, Chemali JJ, Cotten JF, Van Dort CJ, Kim SE, Ba D, Taylor NE, Brown EN, Solt K: Physostigmine and methylphenidate induce distinct arousal states during isoflurane general anesthesia in rats. Anesth Analg 2016; 123:1210–9
13. Solt K, Cotten JF, Cimenser A, Wong KF, Chemali JJ, Brown EN: Methylphenidate actively induces emergence from general anesthesia. Anesthesiology 2011; 115:791–803

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