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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 study¹ 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_ARs), including site-specific interactions. Our demonstration of “antagonistic activity” by flumazenil on GABA_ARs 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_AR. 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_AR.

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 non-competitive 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_AR 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_AR 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 agonists⁷ and antagonists⁸ are applied by microdialysis to the retrotrapezoid nucleus in the ventral medulla. As discussed above, flumazenil does not affect the GABA_AR 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 ($\text{pH} = 7.398$) 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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