INFLUENZA

Response to comment on "Antibodies to influenza nucleoprotein cross-react with human hypocretin receptor 2"

Lawrence Steinman[1]* and S. Sohail Ahmed[2]*

Vassalli *et al.*'s study does not involve or provide additional data regarding influenza virus, influenza vaccines, human samples, animal models of narcolepsy, or experiments related to mimicry and cross-reactivity. They present data on the distribution of hypocretin (HCRT) (also known as orexin) receptors in the brain of an engineered mouse developed by them.

They generated Cre/lox mice using a reporter strain expressing enhanced green fluorescent protein (eGFP) to track the expression of HCRT receptors 1 or 2. Brain sections from these mice were then labeled using antibodies to HCRT and visualized by immunofluorescence microscopy for colocalization. The microscopy studies led them to conclude that mouse HCRT neurons do not express either HCRT receptors 1 or 2. These findings, as they indicate, contrast with the transgenic mouse studies published in 2010 by Yamanaka *et al.* (*1*) who demonstrated direct and indirect activation of HCRT neurons by HCRT through the HCRT receptor 2. Vassalli *et al.* state that these studies conducted in 2010 may reflect the lack of specificity of transgenic mice because not all eGFP-immunoreactive neurons were HCRT-positive. Although this level of granularity is accurate, we believe that Yamanaka *et al.*'s study merits clarification to enable the reader to fully interpret Yamanaka *et al.*'s findings and compare them with those presented by Vassalli *et al.*

The eGFP-expressing transgenic mouse studies conducted by Yamanaka *et al.* tracked the expression of HCRT-producing neurons. These transgenic mice enabled them to conduct sophisticated electrophysiological studies at the HCRT neuron level that measured whether these neurons could be depolarized with the application of either HCRT-1 or HCRT-2 peptides (also known as orexin A and orexin B) in the presence or absence of glutamate receptor antagonists to assess both indirect and direct activation. Depolarization by one (or the other) HCRT peptide would suggest the presence of an HCRT receptor on these HCRT-producing neurons and indeed demonstrated the direct activation of HCRT-producing neurons by HCRT-2 peptide, implicating HCRT receptor 2. HCRT was also demonstrated to indirectly activate HCRT-producing neurons through activation of glutaminergic neurons, but it had little effect on GABAergic neurons directly innervating HCRT-producing neurons. To confirm the receptor specificity of the abovementioned findings, Yamanaka *et al.* additionally bred knockout mice from these eGFP-expressing transgenic mice that systemically lacked either HCRT receptor 1 or 2. Again, electrophysiological studies conducted at the HCRT-producing neuron level demonstrated that HCRT-2 induced depolarization in HCRT-producing neurons of HCRT receptor 1 knockout mice was similar to wild type but failed to induce similar depolarization in HCRT-producing neurons from HCRT receptor 2 knockout mice, indicating that HCRT receptor 2 was the primary receptor for this response.

[1]Neurology and Neuroscience, Stanford University School of Medicine, Stanford, CA 94305, USA. [2]Global Clinical Sciences, Novartis Vaccines Srl, Siena 53100, Italy.
*Corresponding author. E-mail: steinman@stanford.edu (L.S.); sohail.q.ahmed@gsk.com (S.S.A.)

Finally, Yamanaka *et al.* additionally used transgenic mice whose orexin neurons expressed a light-activated chloride pump halorhodopsin (haloR) fused with GFP to conduct detailed immunoelectron microscopic analyses along with three-dimensional reconstruction of the HCRT-producing neurons to visualize the entire cell morphology. These studies demonstrated direct contacts among HCRT-producing neurons, suggesting that connections between HCRT-producing neurons form a positive feedback circuit. Using commercial antibodies against orexin, they noted that "almost all" orexin-immunoreactive neurons expressed both haloR::GFP and eGFP, but no ectopic expression of GFP other than orexin-immunoreactive neurons was observed. Although Vassalli *et al.* are correct that "not all eGFP-immunoreactive neurons were HCRT-positive" in the Yamanaka *et al.* studies, the lack of ectopic expression of GFP and additional confirmatory electrophysiologic studies conducted by Yamanaka *et al.* are difficult to ignore. Thus, we do not agree with Vassalli *et al.* that the conclusions drawn by Yamanaka *et al.* are based on experiments that "lack specificity."

On the topic of "specificity," Vassalli *et al.* also indicated that the commercially available HCRT receptor 2 antibody used in our study lacks specificity. Again, similar to the statement regarding Yamanaka *et al.*'s work, we believe a clarification is merited. First, the HCRT receptor 2 antibodies used for our studies were purchased from Abcam and were generated using a peptide from the first extracellular domain of the human HCRT receptor 2 as the immunogen. Second, this antibody against the extracellular domain of human HCRT receptor 2 demonstrated the classic membrane "punctate" staining typical for G protein–coupled receptors (*2*, *3*) such as HCRT receptors. Third, preincubation of the commercial antibody with a commercially available recombinant peptide comprising the first extracellular domain of HCRT receptor 2 demonstrated significant inhibition of antibody reactivity to HCRT receptor 2 both in enzyme-linked immunosorbent assay (ELISA) and confocal immunofluorescence microscopy studies. Finally, the cell line used for the ELISA and the microscopy studies was specifically engineered to express the human HCRT receptor 2 on its surface. Therefore, we believe the antibody for HCRT receptor 2 used in our study was specific.

Because the data provided by Vassalli *et al.* contain no studies related to influenza, animal models of narcolepsy, or cross-reactivity/molecular mimicry, we believe it is premature to comment on the relevance of the conclusions they have drawn between their findings in a Cre/lox mouse model and our publication on influenza vaccine–induced narcolepsy. Our findings of antibodies against the HCRT receptor 2 do not preclude a direct modulation of HCRT neurons through an autoreceptor (*1*) or an indirect modulation of HCRT neurons through other neurons

important in sleep arousal that express HCRT receptor 2 and send neural projections back to regulate HCRT neurons (*4*–*6*). One additionally needs to consider that antibodies binding to a receptor may have different effects including (i) inhibition of receptor signaling, (ii) stimulation of receptor signaling, (iii) triggering of programmed cell death, (iv) cellular cytotoxicity, (v) cell clearance by complement-mediated pathways, or (vi) receptor internalization. Needless to say, narcolepsy is a complex spectrum of a sleep disorder whose mechanism is continuously being refined by ongoing effort of experts studying HLA (human leukocyte antigen) genetics (*7*, *8*), cataplexy's link to cerebrospinal fluid hypocretin levels (*9*, *10*), and the complex neural pathways involving HCRT and its receptors (review papers from last 2 years only) (*11*–*14*). Their findings should remind us that just because the data are not 100% black or white, one should not conclude that the data are inaccurate—one should realize that clues have been revealed that we are not yet in the position to understand.

REFERENCES

1. A. Yamanaka, S. Tabuchi, T. Tsunematsu, Y. Fukazawa, M. Tominaga, Orexin directly excites orexin neurons through orexin 2 receptor. *J. Neurosci.* **30**, 12642–12652 (2010).
2. H. Zheng, C. Worrall, H. Shen, T. Issad, S. Seregard, A. Girnita, L. Girnita, Selective recruitment of G protein-coupled receptor kinases (GRKs) controls signaling of the insulin-like growth factor 1 receptor. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 7055–7060 (2012).
3. D. Colas, A. Manca, J. D. Delcroix, P. Mourrain, Orexin A and orexin receptor 1 axonal traffic in dorsal roots at the CNS/PNS interface. *Front. Neurosci.* **8**, 20 (2014).
4. A. Inutsuka, A. Yamanaka, The regulation of sleep and wakefulness by the hypothalamic neuropeptide orexin/hypocretin. *Nagoya J. Med. Sci.* **75**, 29–36 (2013).
5. M.-F. Wu, R. Nienhuis, N. Maidment, H. A. Lam, J. M. Siegel, Role of the hypocretin (orexin) receptor 2 (Hcrt-r2) in the regulation of hypocretin level and cataplexy. *J. Neurosci.* **31**, 6305–6310 (2011).
6. P. O. Valko, Y. V. Gavrilov, M. Yamamoto, H. Reddy, J. Haybaeck, E. Mignot, C. R. Baumann, T. E. Scammell, Increase of histaminergic tuberomammillary neurons in narcolepsy. *Ann. Neurol.* **74**, 794–804 (2013).
7. H. M. Ollila, J.-M. Ravel, F. Han, J. Faraco, L. Lin, X. Zheng, G. Plazzi, Y. Dauvilliers, F. Pizza, S.-C. Hong, P. Jennum, S. Knudsen, B. R. Kornum, X. S. Dong, H. Yan, H. Hong, C. Coquillard, J. Mahlios, O. Jolanki, M. Einen, I. Arnulf, B. Högl, B. Frauscher, C. Crowe, M. Partinen, Y. S. Huang, P. Bourgin, O. Vaarala, A. Désautels, J. Montplaisir, S. J. Mack, M. Mindrinos, M. Fernandez-Vina, E. Mignot, HLA-DPB1 and HLA class I confer risk of and protection from narcolepsy. *Am. J. Hum. Genet.* **96**, 136–146 (2015).
8. M. Tafti, H. Hor, Y. Dauvilliers, G. J. Lammers, S. Overeem, G. Mayer, S. Javidi, A. Iranzo, J. Santamaria, R. Peraita-Adrados, J. L. Vicario, I. Arnulf, G. Plazzi, S. Bayard, F. Poli, F. Pizza, P. Geisler, A. Wierzbicka, C. L. Bassetti, J. Mathis, M. Lecendreux, C. E. H. M. Donjacour, A. van der Heide, R. Heinzer, J. Haba-Rubio, E. Feketeova, B. Högl, B. Frauscher, A. Benetó, R. Khatami, F. Cañellas, C. Pfister, S. Scholz, M. Billiard, C. R. Baumann, G. Ercilla, W. Verduijn, F. H. J. Claas, V. Dubois, J. Nowak, H.-P. Eberhard, S. Pradervand, C. N. Hor, M. Testi, J.-M. Tiercy, Z. Kutalik, DQB1 locus alone explains most of the risk and protection in narcolepsy with cataplexy in Europe. *Sleep* **37**, 19–25 (2014).
9. M. S. Heier, T. Evsiukova, S. Vilming, M. D. Gjerstad, H. Schrader, K. Gautvik, CSF hypocretin-1 levels and clinical profiles in narcolepsy and idiopathic CNS hypersomnia in Norway.*Sleep* **30**, 969–973 (2007).
10. I. O. Ebrahim, R. S. Howard, M. D. Kopelman, M. K. Sharief, A. J. Williams, The hypocretin/orexin system. *J. R. Soc. Med.* **95**, 227–230 (2002).
11. L. Shan, Y. Dauvilliers, J. M. Siegel, Interactions of the histamine and hypocretin systems in CNS disorders. *Nat. Rev. Neurol.* **11**, 401–413 (2015).
12. R. S. Liblau, A. Vassalli, A. Seifinejad, M. Tafti, Hypocretin (orexin) biology and the pathophysiology of narcolepsy with cataplexy. *Lancet Neurol.* **14**, 318–328 (2015).
13. T. Sakurai, The role of orexin in motivated behaviours. *Nat. Rev. Neurosci.* **15**, 719–731 (2014).
14. A. Inutsuka, A. Yamanaka, The physiological role of orexin/hypocretin neurons in the regulation of sleep/wakefulness and neuroendocrine functions. *Front. Endocrinol.* **4**, 18 (2013).

Submitted 1 October 2015
Accepted 19 October 2015
Published 18 November 2015
10.1126/scitranslmed.aad6789

Citation: L. Steinman, S. S. Ahmed, Response to comment on "Antibodies to influenza nucleoprotein cross-react with human hypocretin receptor 2." *Sci. Transl. Med.* **7**, 314lr2 (2015).

Science Translational Medicine

Response to comment on "Antibodies to influenza nucleoprotein cross-react with human hypocretin receptor 2"

Lawrence Steinman and S. Sohail Ahmed

Sci Transl Med **7**, 314lr2314lr2.
DOI: 10.1126/scitranslmed.aad6789

Science Translational Medicine (ISSN 1946-6242) is published by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005. The title *Science Translational Medicine* is a registered trademark of AAAS.