INFLUENZA

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

Anne Vassalli,* Sha Li, Mehdi Tafti*

Did hypocretin receptor 2 autoantibodies cause narcolepsy with hypocretin deficiency in Pandemrix-vaccinated children, as suggested by Ahmed *et al.*? Using newly developed mouse models to report and inactivate hypocretin receptor expression, Vassalli *et al.* now show that hypocretin neurons (whose loss causes narcolepsy) do not express hypocretin autoreceptors, raising questions to the interpretation of Ahmed *et al.*'s findings.

Ahmed and colleagues (*1*) recently reported that "antibodies to influenza nucleoprotein cross-react with human hypocretin receptor 2." This important finding might explain why many cases of child narcolepsy were reported after immunization with the Adjuvant System 03 (ASO3)–adjuvanted A(H1N1)pdm09 influenza vaccine (Pandemrix) during the 2009 flu pandemia, mainly in Finland and Sweden where the highest rates of vaccination were achieved in Europe. Besides several concerns about the report of Ahmed and colleagues (*1*) [for example, 55% of control children were found positive for these autoantibodies as well, lack of availability of peripheral cells and cerebrospinal fluid (CSF), and no evidence of disease transfer in an animal model], a major unanswered question is how these anti–hypocretin receptor 2 (HCRTR2) antibodies may cause the disease. A direct antibody-dependent cell-mediated cytotoxicity appears unlikely as this would result in a massive damage and detectable inflammatory signs because *HCRTR2* is expressed in numerous brain cell types, including some with critical functions (such as histaminergic and serotonergic cell groups) (*2*). A plausible hypothesis might be (if it is shown that these antibodies penetrate the brain) that, similar to myasthenia gravis, anti-HCRTR2 antibodies block and inhibit HCRTR2 function. Such process can lead to narcolepsy symptoms because spontaneous mutations in the *Hcrtr2* gene are found to cause narcolepsy in dogs (*3*), and mice in which the *Hcrtr2* gene was inactivated also present narcolepsy-like symptoms (*4*). In humans, in contrast, both sporadic and, presumably, post-H1N1 cases of narcolepsy with cataplexy are caused by the specific loss of hypocretin (HCRT)–producing neurons. Postmortem examination of narcoleptic brains uncovered extensive loss of these neurons together with the colocalized dynorphin and narp (neuronal activity–regulated pentraxin) neuropeptides in the lateral hypothalamus (*5*, *6*). Also, deficiency in HCRT-1 in the CSF allows specific diagnosis of narcolepsy (*7*). One may hypothesize that HCRTR2 blockade by autoantibodies inhibits HCRT production (HCRT neurons are intact but they do not produce HCRT and colocalized neuropeptides), either by directly binding HCRT neurons or by binding other neurons regulating them. Binding and inhibition of HCRTR2 may thus occur in HCRT-producing neurons themselves. Indeed, Yamanaka and colleagues (*8*) used *Hcrt* gene promoter-driven enhanced green fluorescent protein (eGFP) transgenic mice and reported that hypocretin neurons are critically and directly controlled by HCRTR2, thus arguing that hypocretin neurons express HCRTR2 as autoreceptor. However, these transgenic mice are thought to lack specificity, so not all eGFP-immunoreactive neurons are HCRT-positive. Moreover, commercially available anti-HCRTR2 antibodies [as used in the study of Ahmed and colleagues (*1*)] lack reliability. HCRTR2 expression has thus been difficult to assess at single-cell type resolution, and whether HCRT neurons express HCRTR2 is controversial.

It is noteworthy that in both mouse and dog models of HCRTR2 deficiency, HCRT neurons show normal distribution and HCRT production, and brain HCRT levels appear normal [(*4*, *9*), and Fig. 1E for the mouse model reported here]. Thus, the mechanism by which lesions in HCRTR2 lead to narcolepsy with hypocretin deficiency remains unclear.

We have generated mouse conditional knockout (KO) alleles for *Hcrtr1* and *Hcrtr2*. Our conditional alleles are designed in a way that when Cre-mediated excision within the gene occurs, *GFP* replaces the endogenous protein encoding region (Fig. 1, A and B) and therefore can be used as a reliable reporter for mapping of cells in which these receptors are endogenously expressed (*10*). By using GFP and HCRT double immunohistofluorescence, we found no HCRT/HCRTR2 double-labeled neurons, strongly indicating that HCRT neurons lack HCRTR2 (Fig. 1E). In contrast, our mouse model clearly reveals HCRTR2 expression in histaminergic neurons of the tuberomammillary nucleus (Fig. 1C). Nevertheless, many other hypothalamic (Fig. 1E) and extra-hypothalamic cells are GFP-positive. The identity of many of these HCRTR2 neurons is unknown, and whether they project to and eventually activate (or in any manner affect the activity of) HCRT neurons needs further studies. We found that most hypocretin neurons (302 of 316) also do not express HCRTR1 (Fig. 1F). In conclusion, HCRT deficiency in narcolepsy is most probably due to a specific destruction of HCRT-producing neurons, and although the mechanism (presumably autoimmune attack) remains unknown, there is no evidence that this includes antibodies against HCRTR2. Therefore, until the pathogenicity of the reported anti-HCRTR2 antibodies is established, the findings of Ahmed and colleagues (*1*) should be considered as preliminary and not as evidence for molecular mimicry between H1N1 nuclear protein and HCRTR2, resulting in hypocretin deficiency.

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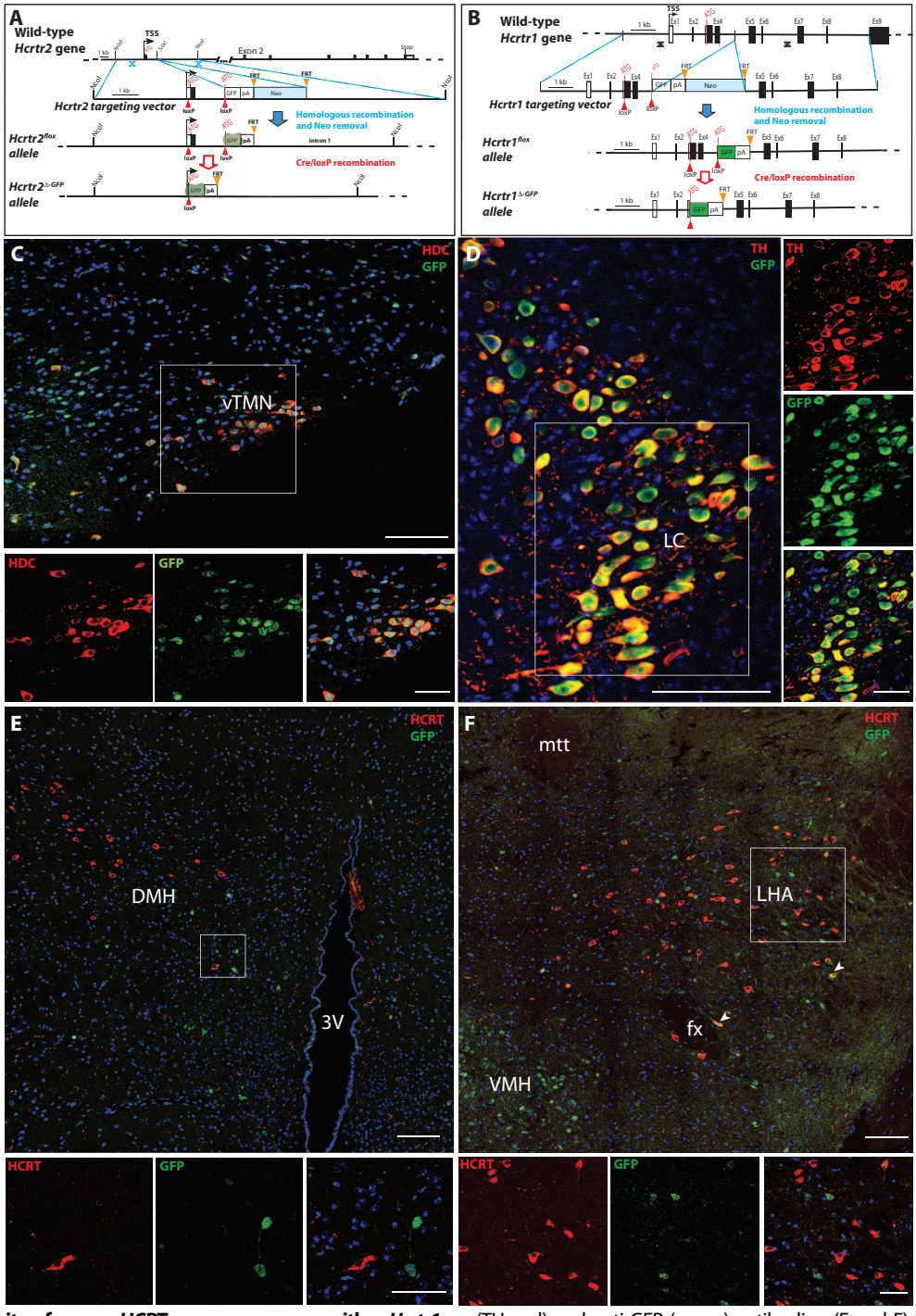

Fig. 1. The vast majority of mouse HCRT neurons express neither *Hcrtr1* nor *Hcrtr2* receptor genes. (**A** and **B**) Schematic representation of *Hcrtr2* (A) and *Hcrtr1* (B) targeting strategy used to generate the conditional KO ("*floxed*") alleles, and, after Neo excision and Cre-mediated recombination, the Δ-*GFP* KO alleles (*10*). (**C** to **F**) Representative confocal micrographs of coronal brain sections from *Hcrtr2*$^{\Delta\text{-}GFP}$ (C and E), or *Hcrtr1*$^{\Delta\text{-}GFP}$ (D and F) mice stained by immunofluorescence. (C) Histamine neurons in the ventral tuberomammillary nucleus (vTMN) of the hypothalamus of a *Hcrtr2*$^{\Delta\text{-}GFP}$ mouse are co-stained with anti–histidine decarboxylase (HDC, red) and anti-GFP (green) antibodies. (D) Norepinephrine neurons in the locus coeruleus (LC) of a *Hcrtr1*$^{\Delta\text{-}GFP}$ mouse are co-stained with anti–tyrosine hydroxylase (TH, red) and anti-GFP (green) antibodies. (E and F) HCRT-immunoreactive neurons (red) in the hypothalamus of a *Hcrtr2*$^{\Delta\text{-}GFP}$ mouse (E), and most HCRT-immunoreactive neurons in a *Hcrtr1*$^{\Delta\text{-}GFP}$ mouse (F), are located in proximity of, but are not themselves, GFP-immunoreactive cells (green), indicating that they do not express HCRT receptors. (E) Depicts a homozygous *Hcrtr2*$^{\Delta\text{-}GFP}$ KO mouse, demonstrating normal HCRT production in absence of functional HCRTR2 receptor. DMH, dorsomedial hypothalamus; fx, fornix; LHA, lateral hypothalamic area; mtt, mammillothalamic tract; TSS, transcription start site; VMH, ventromedial hypothalamus. Blue, DAPI-stained nuclei. Arrowheads in (F) identify isolated HCRT/GFP doubly stained cells. Scale bars, 100 μm [main image in (C) to (F)]; 50 μm [inset in (C) to (F)].

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10. The targeted *Hcrtr1* alleles [official names: *Hcrtr1*$^{tm1.1Ava}$ Mouse Genome Informatics (MGI):5637400 for the floxed conditional KO allele, and *Hcrtr1*$^{tm1.2Ava}$ (MGI:5637401) for the Δ-GFP KO allele] and *Hcrtr2* alleles [*Hcrtr2*$^{tm1.1Ava}$ (MGI:5637402) for the floxed conditional KO allele, and *Hcrtr2*$^{tm1.2Ava}$ (MGI:5637403) for the Δ-GFP KO allele] will be described in further details elsewhere. Figure 1 (C and E) depict the brain of a mouse heterozygous (C), and homozygous (E) for the *Hcrtr2*$^{\Delta\text{-}GFP}$ allele. The cell distribution pattern seen in the homozygous mouse shown in (E) was also observed in four animals heterozygous for this mutation (thus carrying a *WT* functional *Hcrtr2* allele). Figure 1D shows the brain of a mouse homozygous for the *Hcrtr1* floxed allele and carrying a *Dbh-Cre* transgene. Figure 1F depicts the hypothalamus of a mouse heterozygous for *Hcrtr1*$^{\Delta\text{-}GFP}$.

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