Distribution and Postnatal Development of *Gpr54* Gene Expression in Mouse Brain and Gonadotropin-Releasing Hormone Neurons

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Kisspeptin and G protein-coupled receptor 54 (GPR54) are now acknowledged to play essential roles in the neural regulation of fertility. Using a transgenic Gpr54 LacZ knock-in mouse model, this study aimed to provide 1) a detailed map of cells expressing Gpr54 in the mouse brain and 2) an analysis of Gpr54 expression in GnRH neurons across postnatal development. The highest density of Gpr54-expressing cells in the mouse central nervous system was found in the dentate gyrus of the hippocampus beginning on postnatal d 6 (P6). Abundant Gpr54 expression was also noted in the septum, rostral preoptic area (rPOA), anteroventral nucleus of the thalamus, posterior hypothalamus, periaqueductal grey, supramammillary and pontine nuclei, and dorsal cochlear nucleus. No Gpr54 expression was detected in the arcuate and rostral periventricular nuclei of the hypothalamus. Dual-labeling experiments showed that essentially all Gpr54-expressing cells in the rPOA were GnRH neurons. Analyses of mice at birth, P1, P5, P20, and P30 and as adults revealed a gradual increase in the percentage of GnRH neurons expressing Gpr54 from approximately 40% at birth through to approximately 70% from P20 onward. Whereas GnRH neurons located in the septum displayed a consistent increase across this time, GnRH neurons in the rPOA showed a sharp reduction in Gpr54 expression after birth (to ~10% at P5) before increasing to the 70% expression levels by P20. Together these findings provide an anatomical basis for the exploration of Gpr54 actions outside the reproductive axis and reveal a complex temporal and spatial pattern of Gpr54 gene expression in developing GnRH neurons. **(*Endocrinology* 151: 312–321, 2010)**

Kisspeptin and G protein-coupled receptor 54 (GPR54) are now established as key signaling partners in the neural pathway controlling gonadotropin secretion. Unequivocal data demonstrate that kisspeptin neurons directly control the activity of the GnRH neurons (1) and that kisspeptin-Gpr54 signaling plays a prominent role in puberty onset and ovulation (2, 3). However, it seems likely that kisspeptin and/or Gpr54 are also involved in regulating the activity of neurons outside the GnRH neuronal network. Roles for kisspeptin and Gpr54 have been suggested in the process of brain sexual differentiation (4), control of oxytocin secretion (5), and hippocampal excitability (6). Although information is presently accruing on the distribution of kisspeptin neurons and their projections (7, 8), little headway has been made in determining the locations of Gpr54 expression in the brain.

The initial reports identifying the orphan receptor GPR54 provided some information on its distribution within the human nervous system with several brain regions identified by RT-PCR to express GPR54 mRNA (5, 9). Using *in situ* hybridization in the rat, Lee and colleagues reported that Gpr54 mRNA was predominant in limbic brain regions including the hypothalamus, hippocampus, and periaqueductal gray (PAG) (10). How-

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2009-0552 Received May 14, 2009. Accepted October 19, 2009.
First Published Online December 4, 2009

Abbreviations: AHA, Anterior hypothalamic area; GPR54, G protein-coupled receptor 54; LS, lateral septum; MS, medial septum; P25, postnatal d 25; PAG, periaqueductal gray; rPOA, rostral preoptic area; RP3V, rostral periventricular area of the third ventricle; TBS, Tris-buffered saline.

ever, that study provided only four low-power micrographs of hybridized sections, and there has been no detailed anatomical description of *Gpr54* expression in any species to date. More recent Gpr54 mRNA *in situ* hybridization studies have concentrated solely on identifying its presence in GnRH neurons (11, 12).

The issue of defining Gpr54-expressing cells is not trivial because its expression at a cellular level is low (12, 13), and the development of specific antisera with sufficient sensitivity will likely be difficult. One strategy for defining the distribution of low-abundance G protein-coupled receptors has been to use transgenic mice in which reporter molecules such as β-galactosidase have been knocked in to the gene of interest (14–16). By using hemizygous mice that exhibit a normal phenotype, the distribution of cells transcribing the molecule of interest can be assessed with Xgal histochemistry, in the case of the *LacZ* reporter, and coupled with immunocytochemistry to assess expression patterns in specific cell types. This strategy has been used successfully to show that approximately 55% of GnRH neurons are actively transcribing *Gpr54* in adult mice (17).

The present investigation aimed to address two issues: first, to provide a detailed mapping of *Gpr54* gene expression in the mouse brain and, second, to address the postnatal development of *Gpr54* gene expression in GnRH neurons and the brain. The first goal aimed to provide clarity to the potential neuronal networks regulated by kisspeptin/Gpr54 in the central nervous system. The second goal aimed to establish when GnRH neurons begin to express Gpr54 after birth. Immunocytochemical studies show that kisspeptin fibers exist in the vicinity of GnRH neuron cell bodies from postnatal d 25 (P25) onward (18), and we have proposed that the innervation of GnRH cell bodies by kisspeptin neurons of the rostral periventricular area of the third ventricle (RP3V) is the limiting step in the final activation of GnRH neurons at puberty (19). However, dual *in situ* data (12) indicate that Gpr54 transcripts are expressed by GnRH neurons at P18, well before kisspeptin fibers are seen around GnRH neurons. Furthermore, there is a suggestion that kisspeptin-Gpr54-GnRH neuron signaling is involved in the process of brain sexual differentiation in the perinatal period (20). Hence, we were curious to establish precisely when GnRH neurons begin to express Gpr54 after birth and, hence, become potential targets for kisspeptin regulation.

Materials and Methods

Animals

Gpr54-mutant mice (21) were housed under conditions of 12 h light (lights on at 0600 h) with *ad libitum* access to food and water. In this transgenic line, an IRES-LacZ cassette replaces the coding exons for *Gpr54* and allows expression of the *Gpr54* to be visualized by histochemical Xgal staining for β-galactosidase, the product of *LacZ*. Heterozygote mating pairs were established for each line and time mated. Offspring were taken for experiments at birth (within 60 min of delivery), P1 (10–12 h old), P5, P20, and P30 and as adult (>60 d of age). The estrous cycle stage of adult female mice was assessed by vaginal smears, and mice were used for experiments on diestrus. Mice were genotyped by PCR as reported previously (21) before (P20 and older) or after (birth, P1, and P5) death, and hemizygous Gpr54-mutant mice were used. Sex of mice taken at birth, P1, and P5 was assessed by PCR for *Sry*. All experimental protocols were performed under the authority of a United Kingdom Home Office Project License and were approved by the Cambridge Animal Ethics Committee.

Xgal histochemistry and GnRH immunocytochemistry

Neonatal mice

Mice investigated at birth and P1 were decapitated, and their brains were carefully removed and placed in a 4% paraformaldehyde, phosphate-buffered solution (pH 7.6) for 4 h at room temperature. Mice examined at P5 were killed by cervical spinal dislocation and brains removed and treated as above. Coronal sections throughout the forebrain were then cut on a vibratome at 50–60 μm thickness in a 1:3 series. All sections were washed in Tris-buffered saline (TBS) and placed in 2% Xgal solution (2 mM $MgCl_2$; 4 mM $K_3Fe(CN)_6$, 4 mM $K_4Fe(CN)_6$, 4 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactosidase) overnight at room temperature. After TBS washes, 1 set of sections was counterstained with Neutral Red 1% wt/vol and mounted on slides. The remaining two sets of Xgal-stained sections were placed in 3% hydrogen peroxide/TBS solution for 5 min to quench endogenous peroxidase activity and washed three times in TBS. Sections were incubated for 48 h at 4 C in a polyclonal rabbit antibody against GnRH (1:3000; provided by Prof. G. Tramu, University of Bordeaux) (22) in TBS containing 0.3% Triton X-100, 0.25% BSA, and 2% normal goat serum. Sections were then washed and incubated with biotinylated goat antirabbit Ig (1:400; Vector Laboratories, Burlingame, CA) for 90 min at room temperature and then washed and placed in Vector Elite avidin-peroxidase (1:100; Vector) for 90 min at room temperature. Immunoreactivity was revealed using glucose oxidase-reacted diaminobenzidine hydrochloride (DAB). Brain sections were placed on slides and coverslipped using an aqueous mountant Hydromount (HS-106; National Diagnostics).

Peripubertal and adult mice

Mice investigated at P20 and P30 and as adults were anesthetized with 0.05–0.01 ml Dolethal 20% (Vétoquinol, Lure, France) and perfused through the heart with 10 ml (P20) or 15 ml (P30 and adult) of 4% paraformaldehyde, phosphate-buffered solution (pH 7.6). Brains were removed and postfixed in the same fixative solution for 1 h before being cut on a vibratome in the coronal plane as described above, but at 30 μm thickness. Sections were placed in Xgal and two sets immunoreacted for GnRH as described above. Because the neonatal tissue is somewhat fragile, sections from P5 or younger mice were cut at 50–60 μm thickness, whereas older than P5 tissue was cut at 30 μm. Previous studies undertaken on adult tissue has shown that the same Xgal-GnRH neuron dual labeling occurs in 30- and 60-μm-thick sections.

Controls consisted of the use of genotyped wild-type mice at different age groups and the omission of the primary antibody from the GnRH immunocytochemistry protocol. Previous studies have demonstrated the specificity of the GnRH antibody for GnRH1 (22). No Xgal staining was observed in wild-type mice, and no GnRH staining was observed when the primary antibody was omitted.

Analysis

Sets of coronal brain sections were examined on an Olympus BX51 microscope at ×10–40 objective power. Sections from adult male and female mice were matched with plates from the Franklin and Paxinos mouse atlas (23) to provide schematic diagrams of *Gpr54* gene expression in the adult brain. The numbers of Xgal-expressing cells in specific brain regions were assessed across postnatal development by examining Neutral Red-counterstained forebrain sections and scoring them as follows: +, fewer than 10; ++, 10–100; +++, 100–1000; and ++++, more than 1000. Dual-label Xgal-GnRH sections were analyzed by counting all GnRH neurons in two sections at three different levels [medial septum (MS), plate 22; rostral preoptic area (rPOA), plate 27; and anterior hypothalamic area (AHA), plate 32], as detailed previously (24), in each mouse. Brown, diaminobenzidine hydrochloride-labeled GnRH neurons were considered to be positive for Gpr54 if the blue Xgal reaction product was found within the cytoplasm of the soma or proximal dendrite. For each mouse, mean total GnRH neuron counts and GnRH neuron+Xgal values and percentage were generated and used to provide age group means. Statistical analysis comparing between age groups was undertaken using ANOVA with *post hoc* Student-Newman-Keuls for multiple comparisons. Statistical analysis comparing different GnRH neuron populations in the same brains was undertaken with the paired *t* test.

Results

Distribution of Gpr54 in adult mouse brain

Xgal histochemistry revealed a heterogeneous pattern of Gpr54 gene expression in the brain (Fig. 1). The blue Xgal reaction product is found predominantly as one or more dot- or donut-like structures within the cytoplasm of the expressing cell (25–27). The location of the donuts within the cell is not indicative of the subcellular localization of the protein being examined.

The most highly concentrated population of Xgal-containing cells was found in the dentate gyrus of the hippocampus (Fig. 1A) with cells located in the molecular, granule, and polymorphic layers. Elsewhere in the hippocampus, Xgal was detected only in the pyramidal layer of the CA3 (Figs. 1B and 2).

Within the hypothalamus, Xgal was restricted to GnRH neurons (Figs. 2 and 3) and a population of cells located in the periventricular region of the posterior hypothalamus (Fig. 2). Notably, we found no evidence for Xgal in the RP3V or arcuate nucleus. Anatomically, the closest Xgal population to GnRH neurons was found in the lateral septum (LS) and MS (Fig. 2). At rostral levels of the septum, there was a complete intermingling of GnRH- and non-GnRH Xgal-positive cells (Figs. 2 and 3D), whereas more caudally, occasional Xgal-positive cells of the intermediate division of the LS flanked GnRH neurons located in the vertical limb of the diagonal band of Broca and rPOA (Fig. 3B).

Outside of the hippocampus and hypothalamus, Xgal-positive cells were clustered in discrete brain regions throughout the central nervous system. These areas are best viewed on Fig. 2 and, notably, include the anteroventral nucleus of the thalamus, PAG, supramammillary nuclei, interpeduncular and pontine and reticulotegmental nuclei (Fig. 1E), superior colliculus, dorsal tegmental nuclei, dorsal cochlear nucleus (Fig. 1D), and cuneate nu-

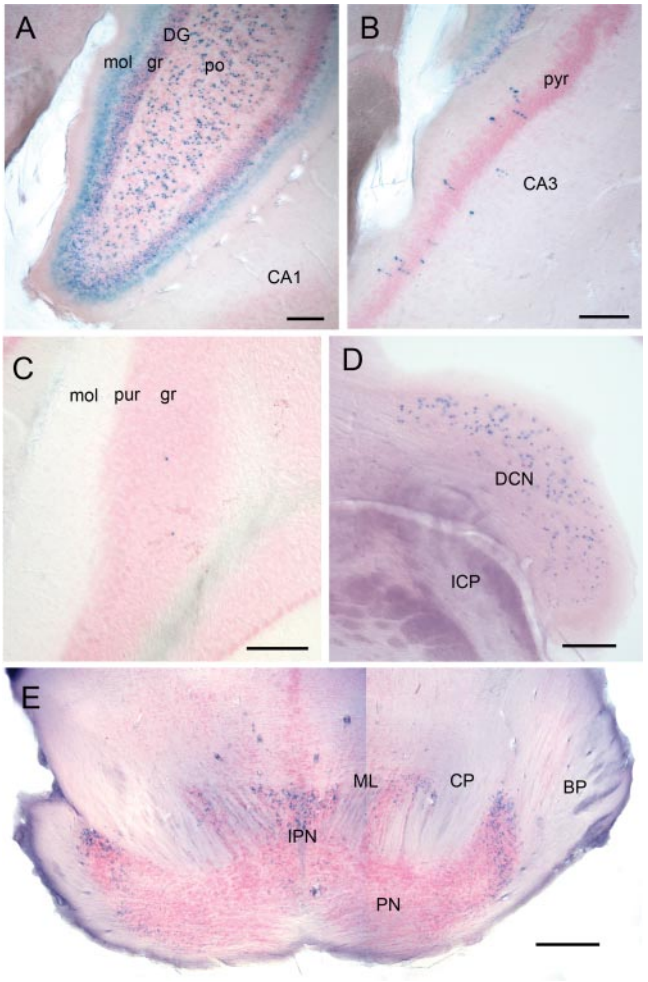

FIG. 1. Distribution of Gpr54-expressing cells in adult diestrous female mouse brain. Photographs show Xgal-expressing cells (*blue*) with Neutral Red counterstain in dentate gyrus of hippocampus (A), pyramidal cell layer of CA3 hippocampus (B), granule cell layer of cerebellum (C), dorsal cochlear nucleus (D), and pontine nuclei (E). BP, brachium pontis; CP, cerebellar peduncle; DCN dorsal cohclear nucleus; DG, dentate gyrus; gr, granule cell layer; ICP, inferior cerebellar peduncle; IPN, interpeduncular nucleus; ML, medial lemniscus; mol, molecular layer; PN, pontine nucleus; po, polymorphic cell layer; pyr, pyramidal cell layer; pur, Purkinje cell layer. *Scale bars*, 250 μm (A and E) and 100 μm (B–D).

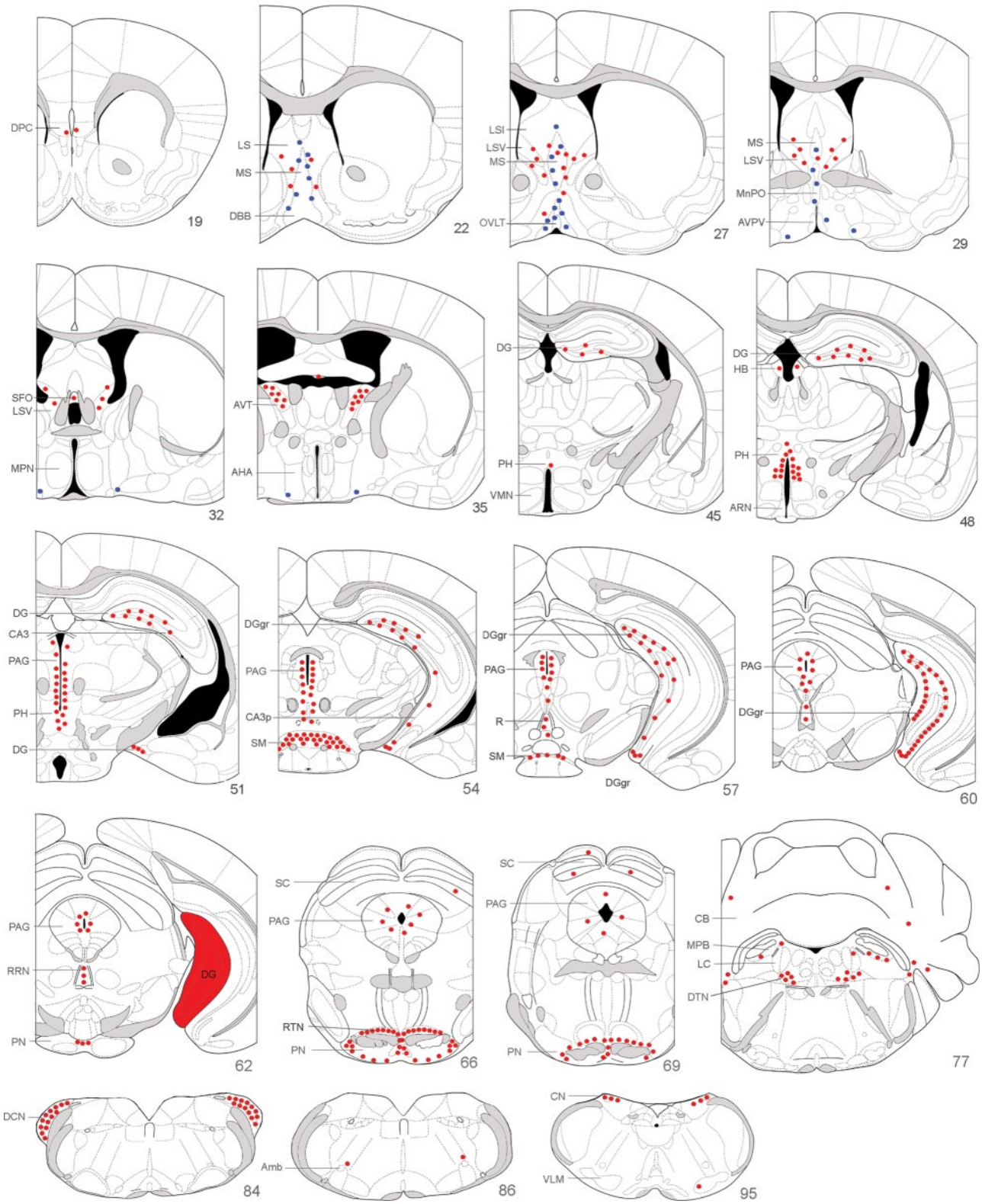

FIG. 2. Schematic diagrams showing distribution of Gpr54-expressing cells throughout the adult female mouse central nervous system. *Red dots*, Locations of Xgal-containing cells. *Blue dots*, Xgal-containing GnRH neurons. The high density of Xgal cells in the dentate gyrus is represented by *solid red*. Diagrams are adapted from Paxinos and Franklin (41) with permission (plate numbers at *bottom right* for each schematic). Amb, Nucleus ambiguus; ARN, arcuate nucleus; AVT, anteroventral nucleus of thalamus; AVPV, anteroventral periventricular nucleus; CA3p, pyramidal cell layer CA3 hippocampus; CB, cerebellum; CN, cuneate nucleus; DBB, diagonal band of Broca; DCN, dorsal cochlear nucleus; DG, dentate gyrus; DGgr, granule cell layer of dentate gyrus; DPC, dorsal peduncular nucleus of cortex; DTN, dorsal tegmental nucleus; HB, habenula; LC, locus coereulus; LSI, intermediate division of LS; LSV, ventral division of LS; MPB, medial parabrachial nucleus; MPN, medial preoptic nucleus; MnPO, median preoptic nucleus; OVLT, organum vasculosum of lamina terminalis; PH, posterior hypothalamus; PN, pontine nucleus; R, raphe; RRN, rostral raphe nuclei; RTN, reticulotegmental nuclei; SC, superior colliculus; SFO, subfornical organ; SM, supramammillary nuclei; VMN, ventromedial nucleus.

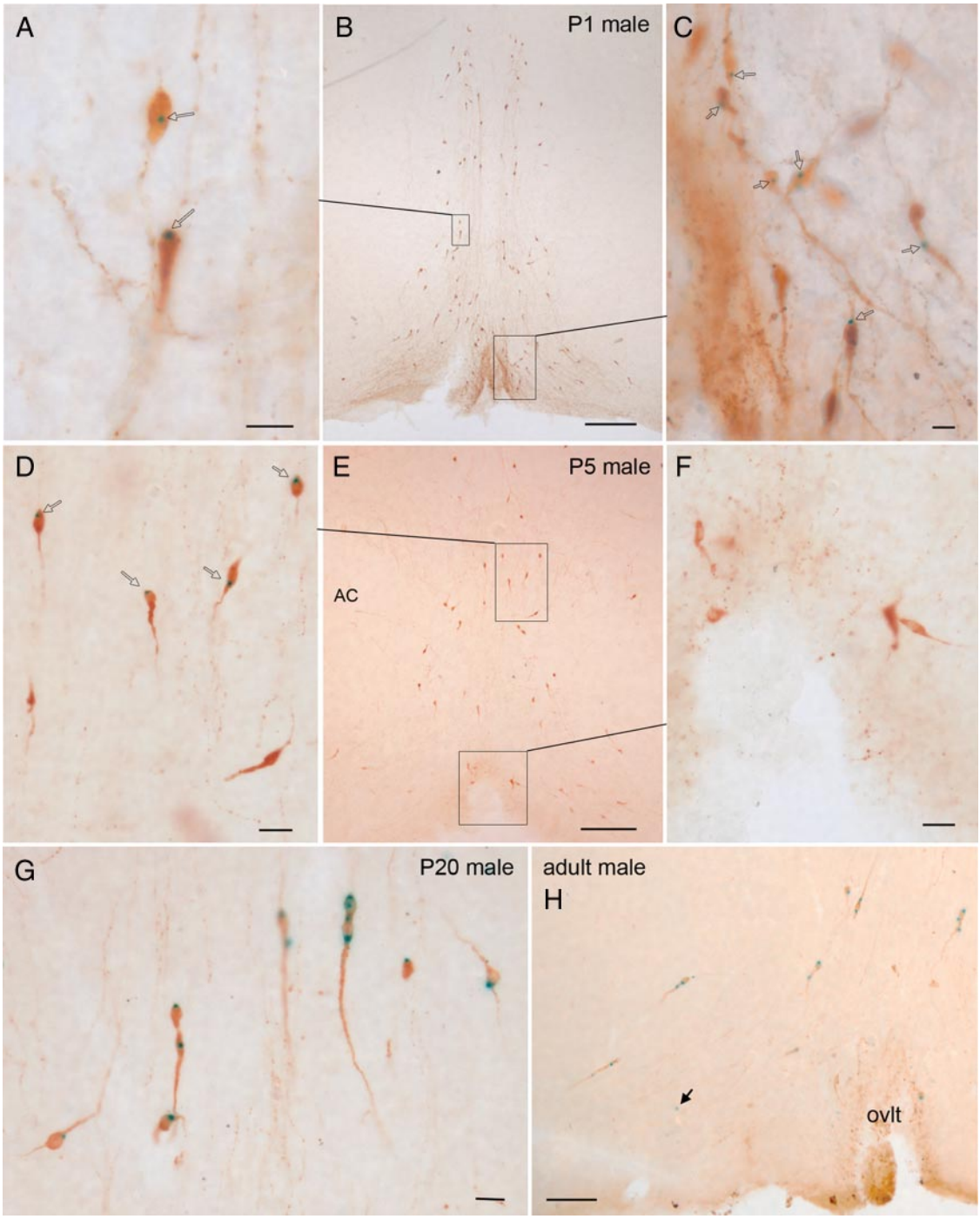

FIG. 3. Dual-label immunocytochemistry showing GnRH neurons (*brown* cytoplasmic staining) expressing Xgal (*blue dots*). A–C, Coronal sections from a P1 male mouse; B, low-power view of the rPOA, and *boxes* indicate the higher magnified views in A and C. GnRH neurons (not all in the plane of focus) with Xgal are indicated by *arrows*. D–F, Coronal sections from a P5 male mouse; E, low-power view of the rPOA, and *boxes* indicate the higher magnified views in D and F. Note that at this age, the ventral GnRH neurons (F) express little Xgal (none in this case) compared with the more ventrally positioned GnRH neurons (D). G, High-power view of GnRH neurons in the medial septum of a P20 male mouse. Note that all GnRH neurons express Xgal. H, Lower-power view of GnRH neurons in the adult male mouse. Note that nearly all GnRH neurons express Xgal in addition to one cell (*arrow*) that is not a GnRH neuron. AC, Anterior commissure; OVLT, organum vasculosum of lamina terminalis. *Scale bars*, 10 μm (A, C, F, and G,), 20 μm (D), 50 μm (H), and 200 μm (B and E).

cleus of the brainstem. A very few Xgal cells were detected in the granule layer of the cerebellum (Fig. 1C), ventrolateral medulla, and subfornical organ.

The distribution of Xgal-expressing cells was examined in both male and female brains and not found to differ.

Postnatal development of Gpr54 in mouse forebrain

Outside of the GnRH neurons, the dentate gyrus and CA3 of the hippocampus were the first brain regions found to express Xgal at P5 (Table 1). In contrast, Xgal was not

TABLE 1. Expression of Xgal in specific forebrain areas across postnatal development from birth to adulthood

	Birth	P5	P20	P30	Adult
GnRH neurons	++	++	++	++	++
Septum	−	−	+++	+++	+++
Hippocampus (dentate and CA3)	−	++	++	++	++++
Posterior hypothalamus	−	−	++	++	+++
Anteroventral thalamus	−	−	+	++	+++
Habenula	−	−	+	+	++
Supramammillary nuclei	−	−	−	+	+++

The numbers of Xgal dots/donuts were recorded for each area: −, no Xgal; +, fewer than 10; ++, 10–100; +++, 100–1000; ++++, more than 1000.

observed in the supramammillary nuclei until P30. In all other brain regions, Xgal was first observed in P20 brains and, as for the other regions, increased in number to reach maximum levels in adults (Table 1). No evidence was found for sex differences or transient expression of Xgal in the forebrain across development.

Distribution and development of Gpr54 expression in GnRH neurons

Immunocytochemistry for GnRH revealed the typical inverted Y distribution of GnRH neurons within the MS, preoptic area, and anterior hypothalamus (28). The numbers of GnRH neurons detected per section were the same in males and females and not different between animals killed at birth, P1, and P5 or between animals killed at P20 and P30 and as adults (Table 2).

Dual-labeled GnRH neurons were identified by the presence of one or two Xgal dots/donuts within their cytoplasm (Fig. 3). Within the rostral hypothalamus, almost all Xgal-expressing cells were found to be GnRH neurons (Fig. 3H). In adult male (n = 4) and diestrous female (n = 4) mice, quantitative analysis showed that 68 ± 5% of all GnRH neurons contained Xgal (Fig. 3H) and that the percentage of expressing cells was independent of their location within the brain (mean percentage in females was 68% in MS, 71% in rPOA, and 51% in AHA and in males was 65% in MS, 59% in rPOA, and 62% in AHA).

Analysis of brains at the different developmental ages showed that GnRH neurons expressed Xgal from the time of birth onward (Fig. 4) but that the percentage of GnRH neurons with Xgal increases with postnatal development. In both males and females, less than 40% of GnRH neurons had Xgal staining in P5 and younger mice (Figs. 3, A–F, and 4) compared with 60–70% of P20 (Fig. 3G), P30, and adult GnRH neurons (Fig. 3H). In female mice, the percentage of GnRH neurons with Xgal was significantly lower in birth, P1, and P5 groups compared with all later stages of development ($P < 0.05$–0.001; ANOVA with Student-Newman-Keuls *post hoc*). Similarly, in male mice, the percentage of GnRH neurons with Xgal was significantly lower in the P1 and P5 groups compared with all later stages of development ($P < 0.05$; ANOVA with Student-Newman-Keuls *post hoc*). The percentage of GnRH neurons with Xgal was not different between males and females at any developmental age point. However, there was a trend for GnRH neurons in P1 females to have very few Xgal-positive GnRH neurons (Fig. 4) with the four individual mice having mean Xgal-GnRH coexpression values of 0, 1, 3, and 47%. The latter mouse was reconfirmed to be female by *Sry* PCR.

In analyzing the patterns of Xgal expression in GnRH neurons, it became apparent that two populations of GnRH neurons existed on an unexpected topographic basis. In individual rPOA sections, an abrupt transition between GnRH neurons with and without Xgal was observed on a dorsal-ventral basis in P1 and, more strikingly, in P5 mice regardless of sex; many MS GnRH neurons had Xgal (Fig. 3D) compared with only very few GnRH neurons located around the organum vasculosum of lamina terminalis (Fig. 3F). This observation prompted a reanalysis of GnRH neurons with dorsal GnRH neurons being defined as those located in the MS and ventral GnRH neurons defined as those located below the level of the anterior commissure at the level of the rPOA and AHA (Fig. 5). This revealed that although Xgal levels in dorsal and ventral GnRH neurons were equivalent at the time of birth, ventral GnRH neurons go on to exhibit a sharp dip in Xgal expression at P1 ($P < 0.01$) and P5 ($P < 0.01$) and that this returns to dorsal levels by P20 (Fig. 5).

TABLE 2. Numbers of GnRH neurons per section in male and female mice at different postnatal ages

	Birth	P1	P5	P20	P30	Adult
Male	33 ± 2	38 ± 4	27 ± 3	18 ± 2	15 ± 2	14 ± 1
Female	32 ± 5	37 ± 2	30 ± 3	18 ± 2	18 ± 1	13 ± 2

Mean ± SEM number of GnRH neurons were counted in six sections (two MS, two rPOA, and two AHA) from each mouse for each group. Note that section thickness was 50–60 μm for birth, P1, and P5 and 30 μm for P20, P30, and adult.

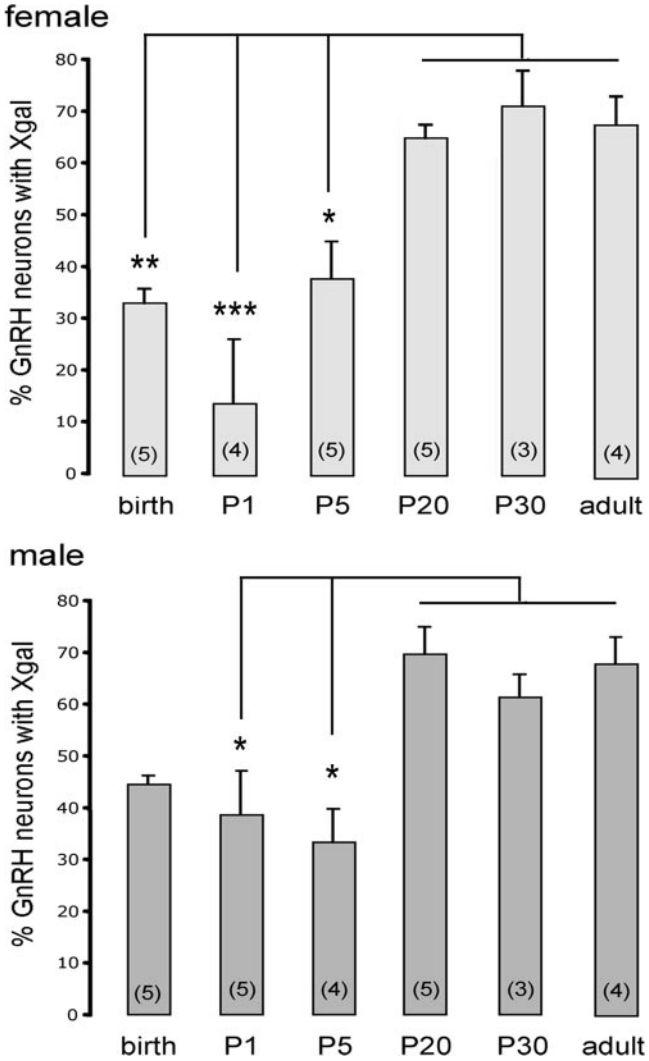

FIG. 4. Numbers of GnRH neurons expressing Gpr54 changes across postnatal development. Histograms show the percentage of female (*top*) and male (*bottom*) GnRH neurons expressing Xgal at birth, P1, P5, P20, and P30 and as adults. The numbers of mice analyzed at each time point are given in *brackets within each bar*. In females, values at birth ($P < 0.01$), P1 ($P < 0.001$), and P5 ($P < 0.05$) were all significantly different compared with P20, P30, and adult values. In males, values at P1 and P5 were all significantly different ($P < 0.05$) compared with P20, P30, and adult values (ANOVA with *post hoc* Student-Newman-Keuls). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

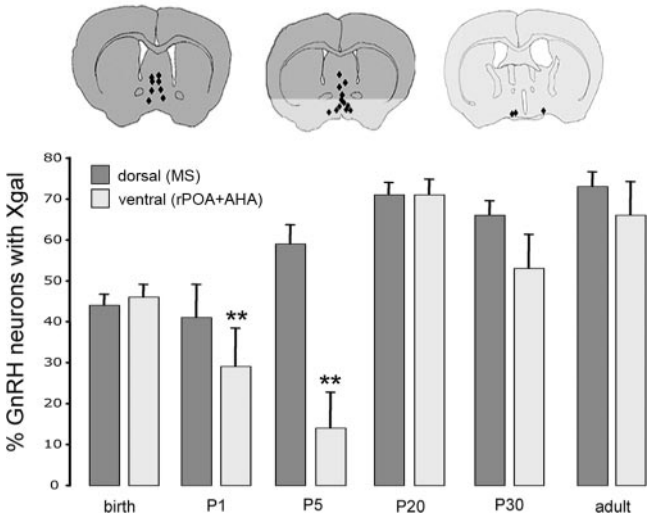

FIG. 5. Numbers of GnRH neurons expressing Gpr54 change depending upon location in the brain across postnatal development. Histograms show the percentage of dorsal (located in MS, *dark gray* in schematics) and ventral (located in the rPOA and AHA, *light gray* in schematics) GnRH neurons expressing Xgal across postnatal development. The numbers of animals (male and female) are the same as that shown in Fig. 4. The percentage of ventral GnRH neurons expressing Xgal was significantly ($P < 0.01$) lower than dorsal GnRH neurons at P1 and P5 (paired *t* test). **, $P < 0.01$.

Discussion

We demonstrate here the distribution of *Gpr54* expression in the mouse brain using a transgenic knock-in strategy that reports on cells transcribing *Gpr54*. In adult mice, *Gpr54*-expressing cells are located in 14 discrete brain regions with the largest population encountered in the dentate gyrus of the hippocampus. Within the hypothalamus, two populations of *Gpr54*-expressing cells were found; the GnRH neurons and a population located in the posterior periventricular hypothalamus. We found that approximately 40% of GnRH neurons expressed Gpr54 at birth and that both spatial and temporal changes in Gpr54 expression by GnRH neurons occurred over the postnatal period.

The knock-in *LacZ* strategy used here has been employed previously to define the distribution of low-abundance G protein-coupled receptors in the mouse brain (15, 16, 29). Because hemizygous Gpr54 mutant mice have a normal phenotype (21), the expression pattern reported here should represent that of a normal mouse. A previous study has used *in situ* hybridization to map the distribution of Gpr54 mRNA expression (10) in the rat brain. All of the brain regions identified here in the mouse to express Gpr54 were also found in rats, including the dentate gyrus, habenula, posterior hypothalamus, PAG, and premammillary nuclei. However, the rat study also identified Gpr54 mRNA in regions not found in here mice: the hypothalamic arcuate nucleus, zona incerta, amygdala, and ventral tegmental area. Although the limited depiction of hybridized sections in that study did not allow precise anatomical definition, it is apparent that some discrepancy exists between these two mapping studies.

It remains curious that cells in brain regions like the arcuate nucleus and RP3V are not transcribing *Gpr54* in the mouse. These areas contain large numbers of kisspeptin fibers in the mouse (7, 18) and rat (30), and Gpr54 transcripts have been reported by RT-PCR in mediobasal hypothalamic dissections or arcuate nucleus punches in the rat (31, 32). Furthermore, Shahab and colleagues (13) provided evidence for low levels of Gpr54 mRNA in the monkey infundibular nucleus using *in situ* hybridization.

At present, it is not clear what underlies these differences; one possibility is that Gpr54 mRNA expression is different in mouse compared with rat and monkey. Another is that Gpr54 gene transcription is below the level of sensitivity for the LacZ-Xgal assay system employed. However, it is also possible that the cells in the RP3V and arcuate do not express Gpr54, and curiously, we have recently observed that kisspeptin-10 has no effects on the electrical activity of RP3V neurons in the mouse (Ducret E., G. Gaidamaka, and A. Herbison, in preparation).

As yet, there is little information on the functions of Gpr54 in brain regions outside the hypothalamus. Arai and colleagues (6) have shown that kisspeptin potently modulates the excitability of granule neurons in the dentate gyrus of the hippocampus. We find here that a very large number of Gpr54-expressing cells exist in all three layers of the dentate gyrus, as well as the CA3 pyramidal cell layer, suggesting a role for Gpr54 in hippocampal functioning. We also note substantial populations of Gpr54-expressing cells in the anteroventral nucleus of the thalamus, PAG, supramammillary and pontine nuclei, and dorsal cochlear nucleus. These distributions would suggest involvement of Gpr54 in cortical relays, learning and memory, nociception, temperature regulation, micturition, cardiorespiratory function, sexual behavior, motor control, and auditory functioning. It is noteworthy that no gross physiological deficits, other than infertility, are reported in Gpr54 mutant mice (21), indicating that Gpr54 signaling in these nonreproductive neuronal networks is not essential and may be modulatory.

A good topographical overlap exists between Gpr54-expressing cells and kisspeptin fiber immunoreactivity in the vicinity of the GnRH neurons, septum, PAG, and posterior hypothalamus of the mouse (7). However, a number of brain regions exist where there are Gpr54-expressing cells but no reported kisspeptin fibers (*e.g.* hippocampus, supramammillary, and pontine nuclei) and, conversely, kisspeptin fibers but no Gpr54 expression (*e.g.* amygdala, hypothalamic supraoptic, and paraventricular nuclei) (7). Although these observations may result from technical issues involving assay sensitivity, they may also indicate that other ligands and receptors exist for Gpr54 and kisspeptin, respectively. It is clear that kisspeptin and GPR54 are essential, exclusive signaling partners for the regulation of reproduction (17, 33), but this may not be the case for other neuronal networks.

Previous studies using dual *in situ* hybridization and electrophysiology have indicated that between 60 and 90% of adult rodent GnRH neurons express Gpr54 (11, 12, 34–36). The present study, using a different approach, found that approximately 70% of GnRH neurons were transcribing Gpr54 in adult mice and that this was not dependent upon sex or anatomical location with the hypothalamus and septum. This agrees with studies that have found similar kisspeptin response rates from septal (36) and rPOA (34) located GnRH neurons and GnRH neurons of both sexes (34). It was notable in the present study that essentially all Xgal-positive neurons in the rostral hypothalamus were GnRH neurons. A small number of Xgal-positive, non-GnRH neurons were found lateral to GnRH neurons in the ventral-most aspect of the intermediate LS. This finding suggests that Gpr54 receptors are expressed exclusively by GnRH neurons within the rostral hypothalamus. Certainly, before P5, the only neurons in the brain transcribing *Gpr54* are GnRH neurons. Indirect effects of kisspeptin on adult GnRH neurons have, nevertheless, been reported in 300-μm-thick coronal brain slices (37) suggesting that neuronal somata or terminals nearby GnRH neurons express receptors responsive to kisspeptin. It is also noteworthy that kisspeptin-Gpr54 signaling can modulate GnRH release from the median eminence (38). The absence of Gpr54-expressing cells in the median eminence itself suggests that any Gpr54 in this area is most likely presynaptic in nature located on GnRH nerve terminals.

We find here that 30–40% of GnRH neurons are actively transcribing Gpr54 at the time of birth. Constantin and co-workers (39) have recently reported that Gpr54 mRNA is found in the nasal region of d-13.5 mouse embryos and that it is likely to be expressed by some GnRH neurons at this time. Together, these studies indicate that subpopulations of GnRH neurons are capable of responding to kisspeptin throughout their prenatal development. The physiological significance of this is not known. The finding here that many GnRH neurons are expressing Gpr54 at birth may be relevant to the recent observation that male Gpr54 knockout mice exhibit female-like sex differences in brain structure (20). Although multiple explanations exist for this phenomenon, one possibility is that kisspeptin-Gpr54 signaling at the level of the GnRH neuron is necessary for early postnatal testosterone secretion required to generate male-like sex differences in the brain (20).

We have uncovered here two patterns of Gpr54 gene expression in postnatal GnRH neurons, the first being an overall increase in the percentage of Gpr54-expressing GnRH neurons across postnatal development and the second being a down-regulation of Gpr54 gene transcription in ventral GnRH neurons after birth. With respect to the former, we had shown previously that the majority of GnRH neurons express Gpr54 mRNA at P18 (12). This is confirmed here and extended to show that the percentage of Gpr54-expressing GnRH neurons increases from ap-

proximately 40% to 70% between P5 and P20. Whether GnRH neurons are exposed to kisspeptin in the early prepubertal period is unknown at present. We have previously suggested that a two-step mechanism of kisspeptin-Gpr54 signaling occurs at the time of puberty in GnRH neurons; this involves a maturation of Gpr54 regulation of GnRH neuron firing (12) and, critically, the expression of kisspeptin in fibers innervating GnRH neurons (18). The finding here that *Gpr54* gene transcription is occurring in adult-like numbers of GnRH neurons at P20 further reinforces the view that GnRH neurons are likely to be sensitive to kisspeptin well in advance of puberty onset.

The rPOA-specific down-regulation of Gpr54 transcription in the first postnatal week is curious and unexpected. Although Gpr54 expression in the dorsal (MS) GnRH neurons exhibits a stable gradual increase across postnatal development, expression in ventral (rPOA/AHA) GnRH neurons falls precipitously in the first postnatal week but recovers to MS levels by P20. Apart from the observation that the rPOA GnRH neuron subpopulation is responsible for the preovulatory GnRH surge in mice, the roles of topographically defined subpopulations of GnRH neurons remain unknown (28). Recent investigations show that GnRH migration occurs in an inside-out manner such that the first-born GnRH neurons are more likely to reside in the MS, whereas later-born GnRH neurons migrate past them into the rPOA (40). However, the roles of MS *vs.* rPOA GnRH neurons in the prepubertal period remain completely unexplored.

In summary, we provide here a detailed description of Gpr54-expressing cells in mammalian brain and show that multiple neuronal networks are likely to use this G protein-coupled receptor from as early as P5. This work provides an anatomical template for the examination of kisspeptin and Gpr54 actions outside the GnRH neuron system. We also show that large numbers of GnRH neurons express Gpr54 from birth and that Gpr54 expression varies in both temporal and spatial dimensions across postnatal development, the physiological significance of which remains unclear at present.

Acknowledgments

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This work was supported by the New Zealand Marsden Fund, United Kingdom Biotechnology and Biological Sciences Research Council Grant BB/C0003861/1, and the Ford Physiology Fund (W.H.C.).

Disclosure Summary: A.E.H., X.d.A.d.T., and W.H.C. have nothing to disclose. J.D. is an employee of Takeda Cambridge.

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