Localization of renin expressing cells in the brain, by use of a REN-eGFP transgenic model

Julie L. Lavoie, Martin D. Cassell, Kenneth W. Gross, and Curt D. Sigmund. Localization of renin expressing cells in the brain, by use of a REN-eGFP transgenic model. Physiol Genomics 16: 240–246, 2004. First published November 18, 2003; 10.1152/physiolgenomics.00131.2003.—Immunoreactive renin has been reported in the hypothalamus and cerebellar cortex in the rodent brain and in neurons in all areas of the human brain. Despite these observations and the clear documentation of the expression of the other renin-angiotensin system genes in the brain, the notion that renin is endogenously expressed in the brain remains very controversial and undefined. This controversy no doubt arises because the level of renin expression in the brain is below the detection threshold of most standard assays. A transgenic mouse expressing enhanced green fluorescence protein (eGFP) under the control of the mouse renin promoter was recently reported. This model expresses eGFP in the kidney, which responds appropriately to both developmental and physiological stimuli. We therefore used eGFP as a sensitive marker to identify renin-expressing cells in the brain. We identified eGFP-containing cells in specific areas of the brain, including cerebellum, hippocampus, dorsal motor nucleus of the vagus, inferior olivary nucleus, reticular formation, rostral ventrolateral medulla, central nucleus of the amygdala, lateral parabrachial nucleus, mesencephalic trigeminal nucleus, bed nucleus of stria terminalis, and subfornical organ. By colabeling with neuron- or glia (astrocytes or oligodendrocytes)-specific antisera, we have determined the eGFP-positive cells to be mainly neuronal. These findings therefore strongly support the primary expression of renin mRNA in the brain in regions controlling cardiovascular function.

ACE/angiotensin receptors, gene expression, genetically altered mice; renin; brain; neuron; astrocyte; oligodendrocytes

The systemic renin-angiotensin system (RAS) is well-known for its effects on blood pressure and fluid homeostasis (for review see Ref. 25). It is now well-recognized that stimulation of brain angiotensin receptors (AT) can contribute to these effects by regulating drinking, salt appetite, vasopressin release, and sympathetic nerve activity (for review, see Ref. 1). Although neither renin nor angiotensin peptides can pass readily through the blood-brain barrier, the presence of AT receptors in circumventricular organs, which are devoid of a blood-brain barrier, permits the influence of circulating angiotensin II (ANG II) on the brain. It has become increasingly appreciated that a local RAS is present in the brain that is involved in the regulation of blood pressure and fluid homeostasis through the stimulation of specific AT receptors (for review, see Ref. 19). However, it remains unclear how ANG II, the physiologically active component of this system, is formed within the brain.

The classic RAS enzymatic cascade involves the cleavage of angiotensinogen by renin to form angiotensin I (ANG I) which is further processed by angiotensin-converting enzyme (ACE) to form ANG II. Although most components of the RAS have been found to be widely distributed in the brain, there are only a few studies demonstrating the presence of renin or renin mRNA. In the early 1970s Ganten et al. (9) and Fischer-Ferraro et al. (6) reported the presence of a renin-like enzyme in dog and rat brain homogenates. Later, renin protein and mRNA were detected in rat (2, 4, 5, 28), human (10), and mouse (5, 32) brain. In spontaneously hypertensive rats, renin has been detected in many nuclei in the brain (2). In addition, although positive renin staining has been found to be present in both neuronal and astrocytic glial cells (14), the specific localization to brain nuclei remains to be elucidated. However, renin expression in brain has remained controversial, because its low level expression makes it extremely difficult to detect in normal animals (see review, Ref. 26). We have reported that ANG II can be formed from renin and angiotensinogen expressed in the brain in transgenic mice (20–23), but clear irrefutable evidence showing production of ANG II in the brain by cleavage of de novo synthesized angiotensinogen from local renin remains lacking. In fact, the formation of ANG II in the brain is further complicated by the postulate that it may be formed by a non-renin-dependent mechanism (11). Questions that clearly remain to be resolved include: What is the regional distribution of renin-expressing cells in the brain? Is renin coexpressed with angiotensinogen or AT receptors? Is renin mRNA expressed in glial cells or neurons?

A novel transgenic mouse model for the study of renin localization has been developed (16). These mice express eGFP driven by 4.1 kb of the renin 5′ flanking region (REN-1c/eGFP). The REN-1c/eGFP mice exhibit tissue-specific expression of eGFP in the kidney and the submandibular gland; at baseline, eGFP expression is highly restricted to the renal juxtaglomerular cells. Moreover, expression of this transgene in the kidney is regulated by the same physiological cues which normally regulate expression of the endogenous renin gene. For example, smooth muscle cells along the afferent arteriole and interlobular artery are recruited to express eGFP following administration of captopril, an ACE inhibitor. The recruitment of renin-expressing cells along the renal arterial tree in response to ACE inhibition has been well documented (12). Also, expression of the REN-1c/eGFP construct accurately follows the spatial- and temporal-expression pattern of endogenous renin mRNA and protein during fetal development.
METHODS

Animals. All experiments were carried out on REN-1c/eGFP transgenic mice provided by Dr. Gross’s laboratory and maintained at the University of Iowa. The mice were subsequently bred and maintained by backcross breeding to C57BL/6J. The animals were maintained on a 12:12-h light/dark cycle with standard laboratory diet (LM-485; Teklad Premier Laboratory Diets, Madison, WI) and water ad libitum. Care of the mice used in the experiments met the standards set forth by the National Institutes of Health in their “Guide for the Care and Use of Laboratory Animals.” All procedures were approved by the University Animal Care and Use Committee at the University of Iowa. The mice were screened for gene expression by PCR of tail genomic DNA using the primers previously published (16). Age- and sex-matched nontransgenic littermates were used as controls in all experiments.

Immunohistochemistry. Mice were euthanized by CO₂ asphyxiation and then perfused transcardially with 20 ml phosphate-buffered saline (PBS) followed by 50 ml of 4% paraformaldehyde in PBS. The brain was removed, postfixed at 4°C overnight, and then placed in 30% sucrose solution at 4°C. The following day the brain was frozen and cut coronally (30°) using a Microm cryostat. Brain sections were washed in 0.1 M sucrose solution at 4°C. The sections were then incubated with rhodamine-conjugated anti-mouse monoclonal antibody (1:100 dilution; Chemicon International), to determine colocalization in astrocytes, oligodendrocytes, or neurons, respectively. We also confirmed eGFP expression in a subgroup of animals by using a rabbit anti-GFP monoclonal antibody (1:100 dilution; Chemicon International). The sections were then incubated with rhodamine-conjugated anti-mouse or anti-rabbit IgG (1:200 dilution; Chemicon International), depend-
ing on the primary antibody, at 25°C for 1 h. Slices were mounted on slides and visualized using a Nikon Eclipse E600 fluorescence microscope equipped with a SPOT RT digital camera (Diagnostic Instruments).

Abbreviations for anatomical regions of the brain are provided in Table 1.

RESULTS

The fidelity and sensitivity of eGFP expression in the kidney of REN-1c/eGFP mice (16) suggested that this model would be an effective tool to identify cells in the brain where the renin promoter is active. Essentially, expression of eGFP became a sensitive marker for the identification of renin-expressing cells in the mouse brain. First, brain sections were examined for eGFP expression by taking advantage of its intrinsic fluorescence. Second, because we were concerned over the possibility of confusing background staining caused by lipofuscin production by monoaminergic neurons, we employed a second criteria based on immunofluorescence using antisera against eGFP. Cells were only designated as positive when their identification was satisfied by both stringent criteria. Over 700 coronal sections from different mice were examined.

A summary of the pattern of eGFP expression throughout the brain is tabulated (Table 1) and is shown schematically in Fig. 1. Expression of eGFP was considered positive only in areas where fluorescence was clearly above background such as in the subfornical organ (SFO) (Fig. 2, A and B) and rostral ventrolateral medulla (RVLM) (Fig. 3A) and was confirmed by using a GFP antibody (Fig. 2, C and D, and Fig. 3B). No eGFP or immunostaining was observed in sections from nontransgenic mice (Fig. 2, B and D; Fig. 3, C and D). The regions where eGFP was identified unambiguously are schematically identified as green (eGFP expression) and blue (neuronal eGFP) in Fig. 1. This confirmation by immunofluorescence was important because we could detect small amounts of autofluorescence in sections from nontransgenic animals, possibly due to lipofuscin. Therefore, because we were unable to confirm eGFP fluorescence with certainty in some regions of the hypothalamus, thalamus, and cortex, we labeled them as having ambiguous expression (yellow in Fig. 1).

In the brain stem, renin-expressing cells as identified by eGFP were mainly found in the dorsal motor nucleus of the vagus (DMNX), inferior olivary nucleus, RVLM (Fig. 4B) and mesencephalic trigeminal nucleus (MeV). We found eGFP in the lateral part of the parabrachial nucleus, whereas no fluorescence could be found in the medial part. In the hippocampus, we found eGFP in the pyramidal cell layer of the CA 1–3 region, and in the polymorphic layer of the dentate gyrus (Fig. 5A), although not in the granule cell layer. In its medial extension, eGFP was seen in the SFO. In the amygdala, we found eGFP to be present in the central nucleus as well as in the bed nucleus of stria terminalis (BNST), whereas it was undetectable in the basolateral group. We found eGFP in the

![Fig. 1. Schematic summary of enhanced green fluorescence protein (eGFP) expression. GFP expression in different coronal sections of the brain going from rostral to caudal (A to D) as illustrated by Franklin and Paxinos (7). The distribution of eGFP is color coded by its certainty of expression and colocalization with cellular markers. Strong eGFP expression that could not be clearly colocalized with markers for neurons, astrocytes or oligodendrocytes are green. Strong eGFP that could be clearly colocalized in neurons are labeled blue. Ambiguous expression is illustrated in yellow. Reprinted from Franklin and Paxinos (7), with permission.](http://physiolgenomics.physiology.org/doi/pdf/10.220.33.1/7)
cerebellum but only in the Purkinje cell layer (Fig. 5B) and not in a nontransgenic control (Fig. 5C). Renin-expressing cells could also be observed in scattered cells throughout the medullary and pontine regions of the reticular formation (RF). More ambiguous expression of eGFP was observed in the nucleus of the solitary tract (NTS), supraoptic nucleus, suprachiasmatic nucleus, and median preoptic nucleus. Ambiguous expression was also observed in the hypothalamus, more specifically in the paraventricular and ventromedial nucleus. Ambiguous expression was also detected in the cerebral cortex, more specifically in the piriform and cingular cortex.

Cellular identity was accomplished using well-characterized markers for astrocytes, neurons, and oligodendrocytes. eGFP was found to be coexpressed in neurons, as determined by the colocalization with NeuN immunostaining, in all regions where it could be detected (schematically shown as blue in Fig.

Fig. 2. eGFP expression in the subfornical organ (SFO). Photomicrographs showing fluorescence observed in brain slices in the region surrounding the SFO of transgenic (A and C) and nontransgenic (B and D) mice looking at eGFP (green, A and B) and using a GFP antibody (red, C and D). SFO, subfornical organ; CC, corpus calosum.

Fig. 3. eGFP expression in the rostral ventrolateral medulla (RVLM). Photomicrographs showing fluorescence observed in the RVLM looking at eGFP (A and C) and using a GFP antibody (B and D) in a REN-1c/eGFP mouse (A and B) and nontransgenic littermate (C and D). Pg, nucleus paragiganto cellularis.
1). An example of clear neuronal colocalization in the MeV (Fig. 4A) and RVLM (Fig. 4B) is shown. The exception to neuronal colocalization was the SFO, where the cell type identification of eGFP cells was unclear. We obtained some evidence for expression of eGFP in oligodendrocytes in the RVLM (Table 1), whereas the identification of eGFP expression in oligodendrocytes in the dentate gyrus was less clear (Fig. 5A). There were no eGFP-positive oligodendrocytes in the cerebellum (Fig. 5B). None of the cells in the areas where we found positive eGFP fluorescence were astrocytes, as determined by GFAP staining (Table 1 and Fig. 6).

DISCUSSION

We examined the localization of renin-expressing cells in different regions of the brain using the REN-1c/eGFP transgenic mice employing eGFP as a sensitive marker for renin-expressing cells. Although expression of renin protein (4, 10, 15, 32) and mRNA (5, 28) has been reported in the brain, its presence has remained controversial and its cellular localization undefined because its level of expression is below the threshold detectable by most standard assays. Because the REN-1c/eGFP mice have a very restricted and tightly regulated pattern of renin expression, this supports our contention that...
the expression of eGFP observed in the brain of these mice reflects that of endogenous renin. Although we cannot exclude the possibility that in tissues with lower levels of eGFP expression the promoter may be leaky, we think this is unlikely. In fact, our confidence in the accurate identification of renin-expressing cells is boosted 1) by the stringent use of a criteria requiring positive signals by both immunocytochemistry in addition to the intrinsic fluorescence of eGFP and 2) by the finding that the location of renin-expressing cells is similar to that previously described for angiotensinogen and ANG II (discussed in detail below).

We found renin-expressing cells in the DMNX, inferior olivary nucleus, RF, RVLM, LPB, MeV, central nucleus of the amygdala, BNST, SFO, CA1–3, DGp, and cerebellum. Renin-containing cells have been reported previously to be distributed in the brain in higher concentrations in the pineal gland, the adenohypophysis, and choroid plexus, followed by the hypothalamus, cerebellum, and amygdala (26). Also, lower concentrations seem to be present in the pons, medulla oblongata, thalamus, hippocampus, spinal cord, and cerebral cortex. In this study and previous work, we have found the distribution of renin to be much more restricted (23). As renin-containing cells have been reported in hypothalamus and thalamus (8, 9, 23), it is possible that these areas may exhibit lower levels of renin promoter activity and thus would be more difficult to evaluate using our model. Indeed, we found the eGFP expression in those areas to be ambiguous, because the levels of fluorescence observed were not clearly above the low levels of autofluorescence in the nontransgenic mice and we did not observe significant staining using the GFP antibody. Also, because the eGFP reporter gene gives us an indication of where the renin promoter is active and not where renin protein is actually present, it is possible that studies looking at immunoreactive renin may be finding areas where it is taken up or secreted.

We found eGFP to be present mainly in neurons which is in accordance with the results of Slater et al. (31), who found immunoreactive renin exclusively in neurons in the human brain, and Hermann et al. (14), who reported that renin is present in neuronal cell culture. Although we previously reported showing renin to be present in glia of mice expressing renin from a large P1 artificial chromosome (23), we found no clear colocalization of renin in either astrocytes or oligodendrocytes using a GFAP and CNPase antibody, respectively. However, it is important to point out that some of the areas labeled as ambiguous provided evidence for glial-specific expression.

Importantly, the eGFP distribution that we found matches closely the distribution that has been reported for both angiotensinogen (AGT) and ANG II. Indeed, neuronal AGT has been reported in specific areas where we have detected eGFP, for instance, the cerebellum, SFO, hippocampus, inferior olivary nucleus, and raphe nucleus (34). Nevertheless, we recognize that the vast majority of AGT can be found in glia throughout the brain (24, 29, 33, 34). Similarly, ANG II immunoreactive neurons are exclusively in the SFO, area postrema, amygdala, LPB, NTS, DMNX, BNST, and RF, although no staining was reported in the cerebellum and MeV (18). Taken all together, this suggests that there may be two models for the generation of ANG II in the brain. In the first, which we term the paracrine mechanism, renin secreted from neurons may interact and cleave angiotensinogen secreted from neighboring glial cells to form extracellular ANG II, which then could interact with AT-1 and AT-2 receptors in the brain. The second model, which we term the autocrine mechanism, generates intracellular ANG I from the intracellular actions of renin and angiotensinogen in neurons. The mechanisms by which ANG I would be converted to ANG II and whether that would occur intracellularly or in the extracellular space remains undefined. While clearly provocative, the second model is supported by recent data obtained from us and others reporting the presence of an alternative renin transcript in the brain which translates to an enzymatically active renin lacking the secretory peptide (i.e., an intracellular renin) (3, 17, 30).

Although there is no direct experimental support for a functional intracellular pathway for ANG II generation in vivo, such a pathway has been hypothesized for renin and many other peptide hormone systems (27). We have recently generated and are currently characterizing transgenic mice in which an intracellular renin has been specifically targeted to the brain, and indeed, preliminary results support a role for intracellular renin generation in blood pressure regulation.

ACKNOWLEDGMENTS

We thank Deborah R. Davis for assistance with the mice used in this study. M. I. Phillips served as review editor for this manuscript submitted by Editor C. D. Sigmund.

GRANTS

This work was supported by National Institutes of Health Grants HL-48058, HL-61446, HL-55006 (to C. D. Sigmund), and HL-48459 (to K. W. Gross). We also acknowledge National Cancer Institute Cancer Center Support Grant CA-16056 awarded to Roswell Park Cancer Institute. J. L. Lavoie is the recipient of an American Heart Association Heartland Affiliate Postdoctoral Fellowship. We gratefully acknowledge the generous research support of the Roy J. Carver Trust.

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