A Cre-loxP solution for defining the brain renin-angiotensin system.
Focus on “Targeted viral delivery of Cre recombinase induces conditional gene deletion in cardiovascular circuits of the mouse brain”

M. Ian Phillips
Department of Physiology, University of South Florida, Tampa, Florida 33620

UNLOCKING THE MYSTERY of the brain renin-angiotensin system (RAS) and its role in cardiovascular physiology has been a theme for state-of-the-art research for 30 years. Each time a new, more precise tool becomes available, the problem is revisited. In this release of Physiological Genomics, Sinnayah et al. (24) present a report on a new way to analyze the role of specific genes in specific cardiovascular circuits in the mouse brain using Cre recombinase delivered by viral vectors.

An independent RAS was first proposed to exist in the brain based on radioimmunoassay and enzyme assays (7). The concept was controversial because the biochemistry of the circulating RAS had been well worked out by 1970. The whole cascade was known. Renin from the kidney was the rate-limiting enzyme to specifically hydrolyze angiotensinogen (AGT) in the liver to angiotensin I (ANG I). ANG I, a decapetide, was converted by angiotensin converting enzyme (ACE) to the octapeptide ANG II. ANG II was the active component. End of story. Ganten et al. in 1971 (7) found evidence of angiotensin-forming enzyme in brain tissue, which was presumably renin.

About that time ANG II had become very interesting, physiologically and behaviorally. When ANG II is injected into the brain it elicits a pressor response and, if water is available, a drinking response. However, in both cases there was controversy about whether the effects reflected the actions of the circulating RAS or required a postulated brain RAS. After all, circulating RAS increased blood pressure, and some of the most efficacious treatments for high blood pressure today are oral or systemically delivered anti-ANG II drugs. Drinking behavior obviously requires neural circuits in the brain. It was reasoned that the effects in the brain were isolated from circulating RAS by the blood-brain barrier (BBB). However, there are small areas of the brain which have no BBB, the circumventricular organs (CVOs). The role of these sites was hotly contended (2).

With this background, there has always been a high level of proof demanded for the brain RAS. One of the techniques applied was iontophoresis, which showed that ANG II activated neurons in the brain and in the subfornical organ (SFO) (18). A peptide antagonist of ANG II, saralasin (which was new at the time), inhibited those neurons, suggesting ANG II was a neurotransmitter. Simply injecting saralasin alone into the brain of hypertensive rats (SHR) temporarily reduced high blood pressure (19). This occurred even in the absence of renin when the SHR rats were bilaterally nephrectomized (19).

Immunocytochemistry with ANG II antibodies showed a clear distribution of ANG II-like staining in the hypothalamus and CVOs such as the SFO, organum vasculosum of the lamina terminalis (OVLT), the median preoptic nucleus (MnPO), and the median eminence. The criticism of those results was that the ANG II antibodies used were polyclonal and therefore not definitive. What was needed was actual measurement of the ANG II protein. This was the achieved with high-performance liquid chromatography (6, 20). Brain levels of ANG II were higher than circulating levels of ANG II.

Having now shown that ANG II was in the brain, the next level of proof required not only measuring the protein but demonstrating that it had been synthesized in the brain. To the rescue came what was then a novel technique, Northern blotting, which showed mRNA for AGT in the brain (3). Renin mRNA was also claimed (5). But blots do not show where in the brain the mRNA is located or expressed. Stornetta et al. (26) identified the site of synthesis of AGT in mRNA using in situ hybridization in glial cells in the hypothalamus.

Another new technique at that time was about to shed much more light on the brain RAS. Mendelsohn et al. (12), using autoradiography with pseudocolor imaging, were able to show maps of the distribution ANG II receptor binding sites located in many nuclei of the brain in addition to the CVOs. This proved that there were sites within the brain that had evolved to be activated by ANG II in the brain. Minute injections of ANG II into key brain nuclei showed that ANG II could differentially produce a pressor effect, eliciting drinking, release vasopressin, inhibit the baroreflex and increase sympathetic nervous system activity (11, 22). The realization that there was a separate brain RAS, independent of a circulating RAS, gave rise to the more general concept of local or tissue angiotensins.

An even bigger breakthrough came when Whitebread et al. (27) and Chiu et al. (4) showed that there were two subtypes of the ANG II receptor, AT1 and AT2. From this has sprung not only insight into genes involved in brain function, but also the highly successful angiotensin receptor blockade (ARB) class of antihypertensive drugs.

Cloning the angiotensin receptors has led to insight into signaling pathways and genetic manipulation. With the development of transgenic mice (17), the first transgenic mouse relevant to RAS was one that demonstrated AGT overexpression by a metallothionein promoter (16). This was followed by the transgenic mouse with an AGT overexpression in brain and liver which had high blood pressure (10). But what about renin? Renin exists in only one form in humans, Ren-1, but in two forms in mice, Ren-1 and Ren-2. The data showing renin in the brain with Northern blots were weak (some critics say ghostly). By inserting a mouse Ren-2 gene into a transgenic rat [TGR(mREN-2)27], Mullins et al. (15) reported fulminant hypertension in transgenic rats. These rats developed high
blood pressure, even though circulating ANG II levels are low. The homogenous offspring develop malignant hypertension and high mortality. The heterozygous offspring have all the hallmarks of end organ damage in the heart and kidney associated with extreme hypertension. The hypertension appears to be due to the brain, because it disappears when the brain is anesthetized. In a further series of studies, Sigmund’s group (14) developed transgenic mice that overexpressed human renin and human AGT. With either a glial fibrillary acidic protein (GFAP) promoter or a synapsin-I promoter, to stimulate gene expression in neuron or glial cells, hypertension and increased drinking resulted.

Transgenic animals have been extremely useful in providing researchers with functional genomics insights into the brain RAS. However, transgenic animals must undergo embryonic development, and an inserted gene or a deleted gene that is not embryonically lethal may be compensated for during the development to maturity. This may account for the surprisingly benign effects of AT2 knockout transgenic mouse (8, 9). To overcome this possibility, we have developed gene suppression methods in adult animals using in vivo, RNA-based inhibition. Antisense inhibition of AGT mRNA or AT1R mRNA oligonucleotides delivered to the brain reduces hypertension in SHR (21). Antisense inhibition of AT1R specifically diminishes AT1 receptors in the paraventricular nucleus (PVN) and OVLT (1). Functionally, AT1 antisense inhibited the effects of ANG II in the brain including the pressor response vasopressin release and drinking (13). In the double transgenic mice developed by Sigmund and colleagues (14) to overexpress both human renin and human AGT so that they constantly have high ANG II levels, antisense to AT1R mRNA dramatically reduced the blood pressure.

Still further proof of the brain involvement is needed to prove the precise roles of the multigene RAS. The question of which genes and exactly where in the brain cardiovascular effects are regulated may now be answered with greater precision. Davison and colleagues, who previously demonstrated uptake of vectors into specific locations of the brain (23), report an approach to gene notification in mice with the Cre-lox system (24). Cre, a bacteriophage P1-derived DNA recombinase, serves to recombine sites of loxP sequences. By flanking an exon or coding sequence with loxP sites, a gene can be “floxed.” The floxed gene will function normally until Cre is present. Since Cre recombines the loxP sequences, the floxed segment is pushed out and rendered nonfunctional.

Previous Cre-lox methods required a transgenic mouse with Cre that is expressed only in specific cells and with a cell-specific promoter. What Sinnayah et al. (24) have shown is that they can eliminate cloning of Cre-expressing transgenic and loxP site mice. They drive Cre with a promoter introduced by vectors. They microinject adenovirus (Ad) and feline immunodeficiency virus (FIV) into selective targets such as the SFO and supraoptic nucleus. Another advantage of this system is that FIV targets neuronal expression of transgenes at the site of injection. Ad, on the other hand, transduces glia and neurons. In neurons, it is conveyed retrogradely. Therefore, Ad can reach distant but connected axes of brain neurons. The Cre-loxP system has previously been used successfully to delete a liver-specific floxed human AGT transgene by adenoviral delivery of Cre-recombinase (25). Using microinjections into the SFO and SON of reporter mice, or cerebral ventricular injections, Sinnayah et al. (24) show that they can overcome the lack of promoters of Cre expression and that loxP-flanked genes can be recombined in select brain nuclei of adult mouse. The gene deletion lasts 7 days to 3 wk, long enough to test different physiological and pathophysiological effects. This new approach can be used at any time point in young and old mice and after any surgery or diet restriction. It can be used to delete any genes, but for brain RAS, Cre could be the key to dissecting the functional genomics roles of brain RAS genes in brain development.

REFERENCES