

**Robin L. Davisson**

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## Physiological genomic analysis of the brain renin-angiotensin system

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**Davisson, Robin L.** Physiological genomic analysis of the brain renin-angiotensin system. *Am J Physiol Regul Integr Comp Physiol* 285: R498–R511, 2003; 10.1152/ajpregu.00190.2003.—The brain renin-angiotensin system (RAS) has long been considered pivotal in cardiovascular regulation and important in the pathogenesis of hypertension and heart failure. However, despite more than 30 years of study, the brain RAS continues to defy explanation. Our lack of understanding of how the brain RAS is organized at the cellular and regional levels has made it difficult to resolve long-sought questions of how ANG II is produced in the brain and the precise mechanisms by which it exerts its actions. A major reason for this is the difficulty in experimentally dissecting the brain RAS at the regional, cellular, and whole organism levels. Recently, we and others developed a series of molecular tools for selective manipulation of the murine brain RAS, in parallel with technologies for integrative analysis of cardiovascular and volume homeostasis in the conscious mouse. This review, based in part on a lecture given in conjunction with the American Physiological Society Young Investigator Award in Regulatory and Integrative Physiology (Water and Electrolyte Homeostasis Section), outlines the physiological genomics strategy that we have taken in an effort to unravel some of the complexities of this system. It also summarizes the principles, progress, and prospects for a better understanding of the brain RAS in health and disease.

transgenic mice; gene transfer; blood pressure; heart rate; dipsogenesis; reactive oxygen species; Cre-lox system; hypertension; heart failure

THESE ARE EXCITING TIMES in physiology research. World-wide genome sequencing efforts, evolving technologies for precise manipulations of the mammalian genome, and increasingly sophisticated methods for integrative physiological analyses are converging to allow us to address questions of complex regulatory processes in ways not possible before. New questions, derivative from gene discovery projects, are beginning to emerge. The physiological genomics era has ushered in great promise for increased understanding of how genes are linked to function under normal conditions and how dysregulation of these processes causes disease. Such knowledge has the potential to impact medicine in ways only imagined in previous scientific eras.

Cardiovascular research is one field that has already benefited substantially from application of physiological genomic strategies. New insights into the function of single genes, genetic interactions, and gene expression profiles are beginning to provide clues to the

molecular basis of normal cardiovascular regulation and the pathogenesis of diseases such as hypertension and heart failure (33). One system that has been particularly amenable to this approach and for which important advances have been made is the renin-angiotensin system (RAS). The RAS constitutes a classical endocrine system with the formation of blood-borne ANG II and the activation of specific high-affinity receptors in cardiovascular target organs (91). However, there is an additional level of complexity with this system due to the redundancy of RAS gene expression in a variety of individual tissues. For example, although kidney and liver are the major endocrine sites of renin and angiotensinogen (AGT) synthesis, respectively, both genes have been detected in a number of extrarenal and extrahepatic tissues (26, 41, 115). The identification of so-called tissue RAS, defined by their potential for local generation and action of ANG II, has led to the concept that the RAS serves multiple cardiovascular regulatory roles that are both interrelated yet independent.

One of these tissue RAS, the brain RAS, has long been considered pivotal in cardiovascular regulation and important in the pathogenesis of hypertension and

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heart failure (11, 91). Yet despite more than 30 years of study, the brain RAS remains poorly understood. A lack of correlation between the substrate, enzymes, receptors, and ANG II itself has made it difficult to resolve how the brain RAS functions under normal conditions and in cardiovascular diseases. A major reason for this lack of understanding has been the difficulty in experimentally dissecting the brain RAS at the cellular, regional, and whole organism levels. Recently, we and others developed a series of molecular genetic tools for selective manipulation of the brain RAS, in parallel with technologies for integrative analysis of cardiovascular and volume homeostasis in the conscious mouse. In this review, I will outline one example of a path that we have taken in an attempt to tackle the brain RAS puzzle using a physiological genomic approach. Certainly it should be acknowledged that there are a number of other investigators in the field whose pioneering work has not only influenced our thinking about how to apply this research strategy, but has significantly advanced our understanding of the role of the brain RAS in homeostatic processes (44, 76, 77, 94, 95, 110, 120, 121).

#### THE BRAIN RAS: GAPS IN OUR EXISTING KNOWLEDGE

Early findings that direct administration of ANG II into the central nervous system (CNS) ventricular system or into particular brain nuclei elicited profound cardiovascular and dipsogenic effects suggested the existence of ANG II processing system(s) inside the blood-brain barrier (65, 69). Subsequent identification of all components of the RAS necessary for ANG II production and action (11), along with evidence of ANG II-like immunoreactivity in some neural circuitries (63, 64), led to the hypothesis that locally generated ANG II functions as a neurotransmitter/neuromodulator in cardiovascular regulatory networks (10). Dysregulation of the brain RAS in various animal models of hypertension (100) and heart failure (24), and amelioration of these disorders by central administration of pharmacological or genetic inhibitors of the RAS (11, 45, 100) have further advanced the concept that brain angiotensinergic systems are critical in the maintenance of cardiovascular homeostasis. Indeed, a number of excellent reviews have been written on the topic over the years (44, 94, 119).

Despite multiple lines of evidence on the importance of the brain RAS, there are large gaps in our knowledge. At the whole organism level, the relative contribution of the central vs. the peripheral vascular and extravascular RAS has been difficult to assess due to the direct interfacing of the systems via circulating ANG II acting at brain regions lacking a blood-brain barrier (the so-called circumventricular organs). Within the CNS, how ANG II is produced locally and the mechanisms by which it exerts its actions in central pathways remain largely unresolved. Central to this issue is our lack of understanding of how the brain RAS is organized at the cellular and regional levels.

Although all of the components of the RAS have been identified in the CNS, a complete RAS in single brain cells in situ or even cellular colocalization of AGT and renin have not been reported. Even at the regional level, there is a mismatch in the distribution of the RAS components. Some have a widespread distribution in the brain, whereas others are relatively confined to cardiovascular centers (15, 21, 60, 80). Various RAS mRNA or proteins have been localized to some cardiovascular regulatory nuclei, whereas others are devoid of the RAS (11). Despite more than 30 years of study and evidence that ANG II-immunoreactive material exists in neurons in the brain (86, 96), we still do not know how it gets there and whether it does in fact function as a signaling molecule in cardiovascular regulatory neural networks.

**AGT.** One of the great controversies regarding the brain RAS has been the localization of the only known precursor of ANG II, AGT. Despite relative agreement between laboratories as to the regions of the brain where AGT is expressed (mRNA and protein) (23, 40), there is considerable disagreement as to the cell type. Many studies have demonstrated that AGT is produced by astrocytes (17, 23, 67, 111), but the presence of AGT in neurons has been a matter of debate. Several immunohistochemical studies report AGT in select neuronal populations (40), but an equal number describe the absence of neuronal AGT (23). More recent findings that AGT is synthesized in neurons in culture (114) and is detected in neurons by RT-PCR (105) revitalized the hypothesis that neurons have the potential to produce intracellular angiotensin(s) from endogenous AGT (105). Because discrepancies among earlier studies may have resulted from the lack of tools sensitive enough to detect relatively low levels of neuronal AGT mRNA, a number of novel transgenic models have been developed to help clarify the localization of brain AGT (21, 128, 129). As described below, these models have helped confirm that AGT is expressed in neurons within highly localized regions and serve as important tools for unraveling this complex system. Furthermore, new models have been generated to address the puzzling question of the functional role of the astrocyte-derived AGT (76).

**Renin.** Another major controversy arises over the identification and localization of renin. It is often considered the "missing link" in establishing the complete pathway for ANG II generation in the brain. Reports both identifying and refuting the presence of renin mRNA, immunoreactive renin, or renin activity in the rodent brain have been published (25, 28–30). No doubt this has remained a significant obstacle to resolution of issues related to the brain RAS because renin expression is at or below the detection of most common assays. A further complicating matter is the fact that renin is not obligate for the generation of ANG II from AGT, as other enzymes have been shown to generate ANG II (7, 54, 102).

**ANG II.** ANG II was first identified in brain of nephrectomized animals using HPLC and radioimmunoassay (93). Later immunohistochemical studies re-

vealed that ANG II was localized to circumventricular organs, magnocellular hypothalamic nuclei, the central amygdala, and the nucleus of the solitary tract (63, 65, 124). Interestingly, ANG II immunoreactivity was found predominantly in neurons of these circuitries (86, 96). However, despite the possibility of highly localized AGT-containing neuronal populations, it is clear that astrocytes are the primary source of AGT in the brain (111). Several hypotheses have been put forth to help explain how extraneuronal AGT could be processed to form intraneuronal ANG II. It has been suggested that ANG II is produced extracellularly and taken up by a carrier system into neurons, as part of an ANG II receptor internalization mechanism or by general endocytosis (27). However, this hypothesis has been refuted on the basis of lysosomal degradation (105). Alternatively, it has been suggested that extraneuronal AGT is taken up and processed intraneuronally; however, studies showing a lack of uptake of radioiodinated AGT by dispersed neurons have refuted this hypothesis as well (105).

**Angiotensin receptors.** The effects of ANG II are mediated by G protein-coupled receptors that can be divided into two pharmacological classes, AT1 and AT2 (16, 98, 116, 125). Although both receptor subtypes are localized to the CNS (125), the AT1 receptors appear to dominate in cardiovascular control regions (71). Although it is generally agreed that central angiotensinergic regulation of blood pressure and drinking is mediated by AT1 receptors (71), there is considerable uncertainty about the precise mechanisms and pathways involved. For example, the mismatch between AT1 mRNA, protein, and ANG II-mediated physiological responses in hypothalamic circuitry remains a puzzle. It is well known that magnocellular neurons of the paraventricular (PVN) and supraoptic nuclei (SON) respond to ANG II stimulation with losartan-sensitive electrophysiological and neurosecretory changes (119, 127). Curiously, however, neither AT1 mRNA nor receptor protein are present in the SON, and in the PVN, AT1 receptors (mRNA or protein) appear to be colocalized with parvocellular rather than magnocellular neurons (60, 61). The lack of AT1 or AT2 receptor binding sites in these ANG II-sensitive regions is also a mystery (3, 31, 118), as are the dichotomies concerning the localization of ANG II peptide itself and its receptors. Dense ANG II immunoreactivity has been reported in magnocellular PVN and SON, median eminence, and the neurohypophysial tracts, but none of these sites have been reported to contain AT1 receptor mRNA or protein (91). Further questions arise regarding the distribution and function of the AT1 receptor subtypes AT1a and AT1b. Encoded by two distinct genes but with high homology and indistinguishable binding profiles (3, 125), the subtypes have been difficult to distinguish, both in terms of regional localization and function. As discussed below, recent physiological genomic studies have been helpful in beginning to dissect the complexities of these receptors in central angiotensinergic responses.

## GENETIC DISSECTION OF THE BRAIN RAS

Of the many questions that remain unanswered about the brain RAS, our interest has focused on three main issues: 1) the functional significance of the differential pattern of RAS gene expression in neuronal and glial cell types; 2) the mismatch in the regional distribution of the RAS components; 3) the relative contributions of the central and peripheral RAS in cardiovascular homeostasis. Common to all three of these themes are questions about how ANG II is produced in the brain and by what mechanisms it exerts its actions. We have used these concepts as a guide in developing and implementing a physiological genomics research strategy that strives for *in vivo* dissection of the brain RAS at the cellular, regional, and whole organism levels. Recent advances in a whole host of molecular tools that allow manipulation of the genome (9, 110), in parallel with increasingly sophisticated integrative physiology in the mouse (13, 43, 48), have greatly facilitated this effort.

The types of *in vivo* genetic manipulations that we have employed to address the role of the brain RAS can be broadly categorized as 1) germline transgenesis, 2) somatic gene transfer using viral vectors, or 3) a combination of these two approaches. Both gene overexpression/constitutive activation and gene deletion/inactivation have been achieved using these strategies. This review is outlined along these lines both for organizational purposes and because it reflects the order in which we have employed these tools for achieving increasingly selective manipulation of the brain RAS.

It should be noted that this is by no means an exhaustive review of all of the work in this field. Rather, the goal is more to illustrate ways in which physiological genomics can be applied to complex questions involving a multi-gene, multi-organ system. Much of the work is "in progress," with the tools still under development and yet to be fully deployed. Many of the physiological analyses have just begun. What follows is a summary of principles, progress, and prospects for physiological genomic analysis of the brain RAS.

### *Germline Transgenesis*

The ability to introduce a genetic change into the germline such that it induces lifelong effects in the progeny was a very important advance in biomedical research (106, 113). At the time that we began to use this research strategy to address questions of the brain RAS, a number of interesting transgenic studies in the field had already set the stage. Mullins et al. (82) had shown that insertion of the mouse *Ren-2* gene into the rat resulted in high brain ANG II levels and fulminant hypertension that could be attenuated by central administration of losartan or lesion of the area postrema (4, 75, 104). Ganten and colleagues (6) developed a novel transgenic rat model expressing AGT antisense under the control of a glial-specific promoter. With significant reductions in brain AGT levels, these rats exhibited reduced dipsogenesis to intracerebroventricular infusion of renin, blunted physiological responses

to salt loading, and increased sensitivity to the pressor effects of ANG II injected into the rostral ventrolateral medulla (5, 6, 39).

*Functional divergence of AT1a and AT1b receptors in the brain.* One of our first studies addressed the question of whether there are differential roles for the AT1a and AT1b receptors in the CNS. In the brain as in the periphery, expression of AT1a generally exceeds AT1b, and there is both overlapping and divergent localization of the receptors (47, 50). Because the ligand specificities and signal-effector coupling are virtually identical (12, 53, 101), the functional roles of the receptors were indistinguishable using traditional pharmacological approaches. In collaboration with Coffman and colleagues (20), we used gene targeting in combination with a system for maintaining indwelling catheters in the cerebral ventricles and thoracic aorta of conscious mice to test whether there are differential roles of AT1a and AT1b receptors in the actions of ANG II in the brain (20). Mice with global deletions of the AT1a (AT1a<sup>-/-</sup>) or AT1b (AT1b<sup>-/-</sup>) receptors were produced using homologous recombination in embryonic stem cells (42, 55, 89). First, we found that wild-type mice have similar dose-dependent blood pressure and drinking profiles to intracerebroventricular ANG II as that seen in other species (20). Second, these studies allowed us to conclude that the pressor effects of central ANG II could be ascribed selectively to AT1a receptors. On the other hand, the central dipsogenic actions of ANG II required the presence of AT1b. Classically considered a redundant AT1 receptor, this was the first demonstration of a primary physiological role for the AT1b receptor subtype.

Although these studies revealed that AT1b was the dominant receptor subtype in the drinking responses to central ANG II, the AT1a receptor appeared to have a minor role in this response (20). Interestingly, a small but significant role for brain AT1a receptors in other volume regulatory mechanisms has been demonstrated using these models. Homeostatic responses to osmotic challenges such as dehydration or high-salt diet were altered in AT1a<sup>-/-</sup> mice (80, 81). Furthermore, increased neuronal activation and vasopressin expression were observed in the PVN of AT1a<sup>-/-</sup> mice exposed to dehydration (81). The relative role of AT1b in these various osmoregulatory responses is currently under investigation, and the AT1b<sup>-/-</sup> model should be an important tool in this regard.

The mechanism of functional divergence between AT1a and AT1b in the brain also remains under investigation. Given the nearly identical receptor binding profiles and signal transduction mechanisms, the differential functions could be explained by divergent localization and/or unique neural circuitry for the two receptors. Interestingly, recent studies using subtype-specific mRNA probes and *in situ* hybridization suggest that the distribution pattern of AT1a and AT1b in rostral forebrain, hypothalamic, and brain stem sites is nearly identical (14). Confirmation of these findings in each of the respective null mutants will be interesting and may help to determine whether the receptor sub-

types are colocalized within the same cells in these regions. It should also be noted that, whereas the coding regions for AT1a and AT1b are nearly identical, the upstream regulatory sequences are quite different (53, 101). Indeed, the divergence in function between these two receptors in the brain and the availability of these important mouse models provides a scenario to identify separately the distal mechanisms involved in ANG II-mediated blood pressure and volume regulation.

Finally, it is interesting to speculate about the significance of these mouse findings in the context of human physiology and pathophysiology. It has been known that the physiological actions mediated by AT1 receptors are very similar in rodents and humans; however, it has generally been accepted that there is only a single human AT1 receptor gene (112). Our findings of divergent functions for the two receptors in mice are thus intriguing and could be explained by the separate functions of the receptors in lower mammals becoming merged such that they evolved to be provided by a single receptor in humans. Alternatively, the recent findings of Li et al. (62) demonstrating the complete loss of ANG II-induced dipsogenesis in mice with targeted deletions of both the AT1a and AT2 receptors raise the possibility of an important role of AT2 receptors and/or a synergistic effect of AT1a and AT2 receptors in drinking behavior. Yet another possibility is the presence of other unidentified angiotensin receptors in human brain. Interestingly, recent information derivative from human genome sequencing suggests there is a separate additional AT1 receptor gene (83). Indeed, this provides an even stronger rationale for a better understanding of the localization and function of AT1a and AT1b receptors, and perhaps others, in the brain.

*Brain-selective overexpression of AT1a receptors causes neurocardiovascular dysregulation.* Although studies using the AT1a and AT1b null mutants did help to identify the separate functional roles of these two receptor subtypes in the brain, they did not allow us to definitively address the question of the relative roles of central and peripheral AT1 receptors, because the genes were deleted in all cells and tissues. So next we turned to different transgenic technology that would allow us to more directly target AT1 receptors selectively in the CNS. We constructed a transgene consisting of the full open-reading frame of the rat AT1a receptor driven off of the neuron-specific enolase (NSE) promoter (58). NSE is a distinct isoform of the glycolytic enzyme enolase found only in terminally differentiated neurons and neuroendocrine cells (103). There is >90% shared sequence identity between the rat and mouse AT1a receptor sequences (12). Transgenic animals were generated by microinjection of the construct into fertilized mouse embryos using standard techniques (106), and mice harboring this transgene (NSE-AT1a) exhibited brain-selective expression of rat AT1a receptors at moderate or high levels (58). Extremely low-level expression of the transgene was detected in the adrenal gland in one of the lines; however,

interestingly, it was not detected in any other tissues including peripheral nerves.

Immunohistochemical studies of NSE-AT1a mice revealed widespread distribution of AT1 receptors in neurons throughout the CNS, albeit at varying levels in different regions (58). An example of a region with particularly robust neuron-targeted AT1 receptor levels is the subformal organ (SFO), one of the circumventricular organs thought to be particularly important in central angiotensinergic mechanisms (Fig. 1). Similar immunostaining patterns were detected in a number of other cardiovascular control regions, including hypothalamic regions, brain stem sites, such as the nucleus of the solitary tract and nucleus acumbens, and lamina terminalis structures. However, it should be noted that there also was ectopic expression of the transgene in the CNS, e.g., dorsal motor nucleus of the vagus, basal nucleus of Meynert, and bed nucleus of the stria terminalis (58).

Using similar integrative physiological methods as described above, we determined that NSE-AT1a mice were not hypertensive, a finding that was somewhat surprising given previous studies suggesting that upregulation of AT1 receptors was linked to hypertension in a number of models (35, 36). However, despite the normal resting arterial pressure, NSE-AT1a mice responded with markedly exaggerated pressor and bradycardic responses after intracerebroventricular administration of ANG II (58). Together, these two observations suggest that upregulation of AT1 receptors in the brain does not affect basal blood pressure but rather causes autonomic dysregulation. Indeed, ongoing studies using both pharmacological blockers and direct sympathetic nerve recording suggest an increase in both sympathetic and parasympathetic tone (59). Determining the precise sites and mechanisms involved will be of interest in future studies. Furthermore, the possibility that there is AT1-mediated baroreflex resetting in NSE-AT1a (58) provides an additional scenario in which this model could provide a valuable tool for investigating underlying mechanisms of important neuroregulatory responses.

In addition to these and other ongoing studies in which we and others are using the model to examine the functional consequences of central AT1 receptor overexpression in the context of pathophysiological activation of the endogenous RAS (e.g., 2-kidney, 1-clip hypertension) (57), one of the most exciting prospects for the NSE-AT1a model could be in genetic complementation studies involving the AT1 knockout models

discussed above (42, 55, 89). By breeding the NSE-AT1a mice onto the AT1 null background, we anticipate generating a new model that is devoid of AT1 receptors in all tissues except the CNS. This will allow even greater precision in pursuit of questions regarding the relative contribution of central vs. peripheral vascular and extravascular AT1 receptors in regulation of cardiovascular and volume homeostasis.

*Transgenic models with neuron- and glia-targeted expression of RAS genes.* Another set of unique transgenic models to address important questions concerning the brain RAS has been developed by Sigmund and colleagues (21, 76–79, 128). These models were designed with an eye toward a better understanding of the mechanisms involved in the local production of ANG II in the brain. Given that a number of controversies surrounding the brain RAS likely have to do with a lack of tools sensitive enough to detect low levels of the genes and protein in the CNS, several models with either human renin or human AGT overexpression were generated to more easily map the localization of these components. For example, mice harboring a large genomic segment (encoded on a P1 artificial chromosome) containing the human renin gene along with much of the 5'-regulatory region have been useful in localizing renin gene expression with regard to region and cell-type specificity in the brain (PAC140 and PAC160) (107). With such a large portion of the upstream regulatory sequences included in this transgene, there is increased likelihood of appropriate expression patterns. Furthermore, human renin is more easily detected due to multiple transgene copies, as well as its discernibility from endogenous renin. As demonstrated before, renin protein was associated with both glia and neurons in the brain of these PAC transgenics (77). Also consistent with earlier reports was the localization of renin to a number of cardiovascular control regions such as the hypothalamus and some structures of the lamina terminalis. However, renin-positive staining was also found in sites where it had not previously been identified, including the neurons of the dorsal cochlear nucleus and hippocampus (77). Similarly, renin expression was not detected in sites where it had previously been thought to exist, such as in brain stem nuclei. It should be noted that renin is also strongly expressed and appropriately regulated in the renal juxtaglomerular cells of these transgenics (77).

Several analogous models useful for mapping the distribution of AGT in the brain have also been generated. Transgenic mice harboring the human AGT gene

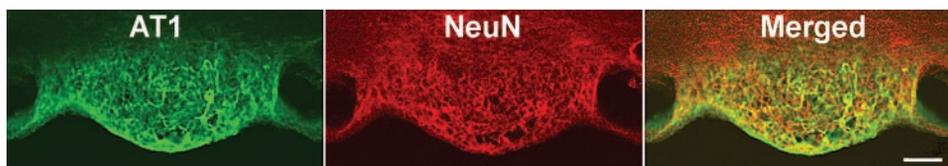


Fig. 1. Neuron-targeted AT1 receptors in the subformal organ (SFO) of NSE-AT1a transgenic mice. Representative confocal images of immunohistochemical staining of the SFO dually stained for AT1 receptors (green) and the neuronal marker neuronal nuclei (NeuN) (red). Double labeling is shown in the merged (yellow) image. Bar = 20  $\mu$ m.

under the control of a relatively large portion of its own endogenous promoter exhibit AGT expression not only in astrocytes in many regions throughout the brain, but also in neurons within highly localized areas of the CNS (21, 128). Interestingly, these sites, including the SFO, paraventricular nucleus, lateral parabrachial complex, and mesencephalic trigeminal nucleus have each been linked to cardiovascular and/or osmoregulatory responses (46). This cellular and regional AGT localization pattern was confirmed in a separate transgenic reporter model in which a  $\beta$ -galactosidase gene was driven by the human AGT promoter (128). Although there had been some suggestion of neuronal AGT expression in a number of reports over the years (40, 115), this finding of highly discrete AGT-positive neuronal populations provided important data suggesting a possible scenario for intraneuronal ANG II production. Similar to the renin model, the human AGT transgenics also exhibit high-level but appropriate AGT expression in other tissues such as the liver (129).

To begin to unravel the functional significance of these complicated patterns of neuronal and glial expression of renin and AGT, these and other human renin and human AGT models have been cross-bred to generate double transgenics. This is necessary to reconstitute a functional human brain RAS capable of producing ANG II, because there is a unique species specificity of the reaction between renin and AGT (human renin does not cleave mouse AGT and vice versa). One of the first such double transgenic models in which the brain RAS was shown to have an important role in blood pressure regulation was the one in which the human AGT mice described above were bred to mice containing a human renin transgene similar to that of the PAC mice but considerably smaller (21). These so-called R+/A+ mice have elevated plasma ANG II levels and sustained hypertension (72). We showed that intracerebroventricular administration of the AT1 receptor blocker losartan restored blood pressure to near-normal levels, suggesting that overproduction of ANG II in the brain of these mice was involved in the maintenance of elevated blood pressure (21). This was associated with increased sensitivity to peripheral vasopressin receptor blockade as well as enhanced plasma vasopressin levels (21).

While this study lends strong support to the notion that overactivation of the brain RAS is involved in the pathogenesis of hypertension, the R+/A+ model exhibits alterations in both the endocrine and tissue RAS. To gain greater regional and cellular specificity for RAS transgene expression in the brain, Sigmund and colleagues (76, 79) subsequently went on to generate a series of double transgenics with glia- or neuron-targeted human renin (various transgene configurations) or human AGT expression in multiple combinations. The synapsin-I promoter has been used to drive expression of human renin or human AGT selectively in neurons (79), whereas astrocyte-restricted expression of each of these transgenes was achieved using the glial fibrillary acidic protein (76). In both cases, the trans-

genes were indeed restricted to the predicted cell type, and expression was generally localized to the CNS. Furthermore, within the brain, there was widespread distribution of the transgenes as expected with these promoters. When the various cell type-specific single transgenics were cross-bred with each other, these investigators found overall that blood pressure was affected relatively modestly (78), if at all (79), in the various double transgenics. However, interestingly, volume and osmoregulatory responses such as salt appetite and water intake were more significantly affected (78, 79). These results suggest that local overproduction of ANG II in the brain leads to dysregulation of volume regulation and further suggests the importance of the brain RAS in body fluid homeostasis.

Full realization of the potential for this array of unique new tools will require further extensive integrative physiology coupled with additional cellular and molecular biological studies. Such as with many transgenic models, the work has just begun. For example, the long-sought questions of whether renin and AGT are coexpressed at the regional and cellular levels and whether this results in local ANG II production could be addressed using some of these models. The functional significance of the complex, differential pattern of renin and AGT gene expression with regard to a whole host of neurocardiovascular regulatory systems will also be amenable to dissection with these models.

#### *Somatic Gene Transfer*

These various transgenic and knockout rodent models in which the brain RAS has been manipulated will be important, especially in pursuit of questions of the relative role of central and peripheral angiotensinergic mechanisms. However, a more refined analysis of the mechanisms and regulation of ANG II-mediated responses in specific brain regions has been more challenging because promoters for restricting gene expression to relevant RAS-containing sites have yet to be identified. One powerful complementary strategy for selective targeting of genes to particular brain regions is through the use of replication-deficient recombinant viral vectors. These have become extremely important tools for gene delivery to the CNS and other tissues in recent years (18) and appear to hold promise for molecular dissection of the brain RAS.

*Gene transfer to a key cardiovascular network in the brain: comparison of two viral vectors.* Recent studies by Davidson et al. (18) and others suggest that adenovirus (Ad) and lentivirus, such as the feline immunodeficiency virus (FIV), are among the most promising vehicles for gene transfer to the brain (108). However, much of the work had been carried out in vitro (73, 97, 120) or in cerebellum, cerebral cortex, or striatum in vivo (2, 8, 18, 56, 84, 99). We recently undertook a series of studies to evaluate the potential of these vectors for in vivo targeting of genes to sites and networks relevant to the brain RAS.

We decided to focus initially on the neural circuitry depicted in Fig. 2. In addition to being critically in-

involved in cardiovascular and volume homeostasis, much of the interest and controversy surrounding the brain RAS have involved this SFO-hypothalamic axis and related nuclei (44). The SFO, one of the circumventricular organs, is thought to couple blood-borne ANG II with neural networks that trigger endocrine and autonomic responses involved in regulating blood pressure and fluid balance (45). The PVN and SON, containing magnocellular vasopressinergic neurosecretory cells, receive direct projections from neurons of the SFO (74). The SFO also innervates parvocellular neurons of the PVN, which project to sympathetic outflow centers in the rostral ventrolateral medulla (RVLM) and intermediolateral cell column of the spinal cord (37). As described above, the RAS has long been implicated as a pivotal mechanism in these circuitries; however, the precise mechanisms remain unknown. In addition to regional mismatches between the various RAS components along this axis (see above), there is a complex pattern of cell-specific expression of the genes. For example, AGT is expressed in both neurons and glial cells of the SFO and SON (21, 128), whereas ANG II receptors are expressed exclusively within neurons in these regions (15, 60, 80). We reasoned that both cell- and site-selective targeting of this circuitry with genes that modify the RAS would be extremely helpful in dissecting out the complexities of this system.

In striatum and other brain regions, Ad and FIV have been shown to exhibit unique properties with respect to cell-type specificity, stability, and infectivity of distant sites through retrograde transport (18, 32, 99). Using replication-deficient Ad and FIV vectors encoding a  $\beta$ -galactosidase reporter gene (Ad- $\beta$ -gal and FIV- $\beta$ -gal) in conjunction with stereotaxic microinjection, we compared these two vectors for gene transfer to the SFO-hypothalamic axis in mice (108). We focused on this species because of our long-term goal of using virally mediated gene delivery in transgenic mouse models (see below). First, we demonstrated that tiny nuclei in the mouse brain such as the SFO and SON could be selectively and efficiently targeted in

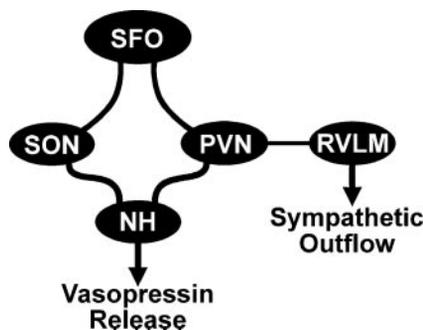


Fig. 2. Schematic of the SFO-hypothalamic axis. SFO is a primary sensor for blood-borne and ventricular ANG II. It sends projections to the vasopressin-synthesizing magnocellular neurons of the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei, which in turn descend to the neurohypophysis (NH) where vasopressin is stored for release into the circulation. SFO also innervates parvocellular neurons of the PVN, which project to sympathetic outflow centers in the rostral ventrolateral medulla (RVLM).

vivo using either viral vector (108). By placing the injector just dorsal to the structures and using small injection volumes, we were able to induce highly localized gene transfer to the individual sites without damaging them. There was limited diffusion of the viruses from the target region, and the results were robust and reproducible. This represented a major step forward in the ability to achieve brain site-selective gene manipulation.

In this study, we also showed that the two viruses differed markedly with regard to cell-type specificity (108). FIV targeted transgene expression selectively to neurons in these nuclei, presumably by virtue of the pseudotype (vesicular stomatitis virus) of this particular virus (2). In contrast, positive X-gal staining was detected in both neurons and glial cells of brain sites microinjected with Ad- $\beta$ -gal. Given the differential pattern of RAS gene expression in neurons and glia along this axis, the cell specificity of these different vectors is a feature that could be brought to bear on the molecular dissection of this system. For example, microinjection of an FIV vector encoding an AGT-modifying gene selectively into the SFO could help to address the unique functional role of neuronal AGT in this region.

Another property that was distinctly different for Ad and FIV that we hope to capitalize on is the local vs. distant infectivity with these two viruses. We showed that Ad- $\beta$ -gal injected into the SON was taken up by nerve terminals and retrogradely transported back to a subset of neurons in the SFO (108), presumably the cells that send direct short loop projections downstream to the SON (85, 87). Because it is thought that there are functionally distinct groups of cells in the SFO and in other nuclei along this axis (70), Ad could be a very powerful tool for dissecting out the function of genes expressed in unique subpopulations of neurons in these regions. Indeed, one recent study demonstrated successful targeting of magnocellular PVN and SON neurons via pituitary injections of an Ad vector (121).

On the other hand, spatially restricted gene transfer without transduction of secondary regions is also desirable for addressing different sets of questions. Our studies suggested that FIV is the vector of choice for achieving highly localized and stationary transgene delivery to the SFO-hypothalamic axis (108). Additionally, FIV would be more desirable for studies addressing long-term regulation of cardiovascular function. We showed that  $\beta$ -gal activity was sustained over months when it was delivered by FIV, whereas Ad-mediated gene transfer was more transient (3 wk). The stability of FIV-induced gene transfer is likely due in part to the fact that lentiviruses integrate the message into the host genome, whereas adenoviral DNA remains primarily episomal (56).

*Gene transfer to specific brain sites reveals novel ANG II signaling mechanisms.* The power of recombinant viral vectors is already being deployed to study brain angiotensinergic mechanisms in central regulation of cardiovascular function. For example, Phillips and colleagues (92) used intracerebroventricular delivery of an adeno-associated virus encoding antisense

oligonucleotides to AT1 receptors to demonstrate a critical role of the brain RAS in the maintenance of elevated blood pressure in spontaneously hypertensive rats. They showed that antisense-mediated decreases in synthesis of AT1 receptors in the brain of these rats significantly lowered blood pressure for up to 9 wk after injection.

Gene transfer to specific sites in the CNS has also revealed novel mechanisms of ANG II-mediated regulation of cardiovascular function. Paton and colleagues (90) used viral vectors to selectively deliver genes to the solitary tract nucleus (NTS) of a working heart-brain stem preparation in rats. Using an Ad vector encoding a dominant negative mutant of endothelial nitric oxide synthase (eNOS), these investigators demonstrated that ANG II-mediated depression of the cardiac baroreflex in this site involves the activation of eNOS.

We recently used adenoviral-mediated gene transfer to the circumventricular organs of mice to study the intracellular mechanisms by which ANG II mediates its pressor, bradycardic, and dipsogenic actions when administered in the brain (131). Unlike in peripheral tissues where the signaling pathways activated by ANG II binding to AT1 receptors are relatively well established (42), the picture in the CNS is much less clear. Recently a novel signaling mechanism for ANG II involving the generation of superoxide and other reactive oxygen species (ROS) has been identified in tissues, such as the vasculature, heart, and kidney (34, 38, 83). In the brain, overproduction of ROS due to dysregulation of antioxidant mechanisms is implicated in a number of neurodegenerative diseases, including amyotrophic lateral sclerosis and Alzheimer's disease (22, 109). However, very little is known about the role of ROS as signaling molecules in normal neural processes in the brain, including central regulation of cardiovascular function.

To test the hypothesis that superoxide is a key mediator of the cardiovascular and dipsogenic actions of ANG II in the CNS, we used an adenoviral vector encoding the antioxidant enzyme superoxide dismutase (SOD) in normal adult mice instrumented as described above with intracerebroventricular cannulas and intra-arterial catheters (131). Gene transfer of SOD to circumventricular organs such as the SFO abolished the changes in blood pressure, heart rate, and water intake elicited by intracerebroventricular injection of ANG II in conscious mice. Moreover, we showed that neurons isolated from circumventricular organs generate superoxide in response to ANG II *in vitro* and this response is blocked by Ad-SOD transduction of these cultured cells (131). Together, these findings suggest that ROS mediate neuronal responses to ANG II and may be key players in brain RAS-mediated regulation of cardiovascular and volume homeostasis. In ongoing studies we are using a variety of virally encoded transgenes to address the intracellular mechanisms involved in this central redox regulation. For example, preliminary studies using an Ad vector encoding a dominant negative inhibitor of NAD(P)H

oxidase suggest that this complex plays a critical role in the ANG II-mediated generation of ROS in cardiovascular control regions of the brain (130). We are also exploring the possibility that dysregulation of central redox mechanisms is involved in the pathogenesis of diseases such as hypertension and heart failure. Ongoing studies using gene transfer of SOD in mice suggest that overproduction of superoxide in the CNS mediates the neurodysregulation associated with the postmyocardial infarction decline to heart failure (66).

#### *Somatic Gene Transfer + Germline Transgenesis*

As illustrated above, somatic gene manipulation through the use of viral vectors is an important strategy for molecular dissection of complex systems such as the brain RAS. Protein products of desired genes can be overexpressed (e.g., Ad-SOD; 131), downregulated (e.g., antisense; 49), or expressed abnormally such that they inhibit interaction with their usual targets (e.g., dominant-negative mutants; 90, 130). These manipulations can be made to be highly selective because of the spatial and cellular specificity this approach affords. An additional complementary and extremely powerful strategy involves the "overlying" of somatic gene transfer with germline transgenesis. This combined approach of using viral vectors to deliver genes to genetically engineered mice allows for an additional level of control over the spatial, cellular, and even temporal expression of genes. There are a number of applications of this strategy, including local injection of viral vectors to increase a particular gene product in an attempt to "rescue" function in a knockout model. An additional approach involves the induction of tightly restricted gene deletion using the Cre-lox system (117), which is the focus of our current studies.

*Selective Cre-mediated gene deletion in key cardiovascular control regions of the brain.* The Cre-lox system is a particularly exciting application of molecular genetics for manipulating complex systems such as the RAS, with its multiple genes and redundant tissue expression patterns. Cre is a bacteriophage P1-derived DNA recombinase that catalyzes recombination between two appropriately oriented 34-base pair recognition sequences termed loxP sites (52). As illustrated in the schematic in Fig. 3, loxP sites can be inserted into the mouse genome (by homologous recombination in embryonic stem cells) such that they flank important coding sequences of a particular gene of interest (gene "floxed"). Genes appropriately modified to contain loxP sites within introns function normally in the absence of Cre, but are rendered nonfunctional in the presence of Cre (see Fig. 3) (52). Because the floxed gene is found in every cell and tissue of the mouse, the specificity of the gene excision arises from selective delivery of Cre. So far, this has been accomplished primarily by crossbreeding the floxed mouse with a second mouse that harbors a Cre transgene under the control of a tissue/cell-specific promoter. For example, Tonegawa and colleagues (117) were among the first to use the system in the brain and showed hippocampal

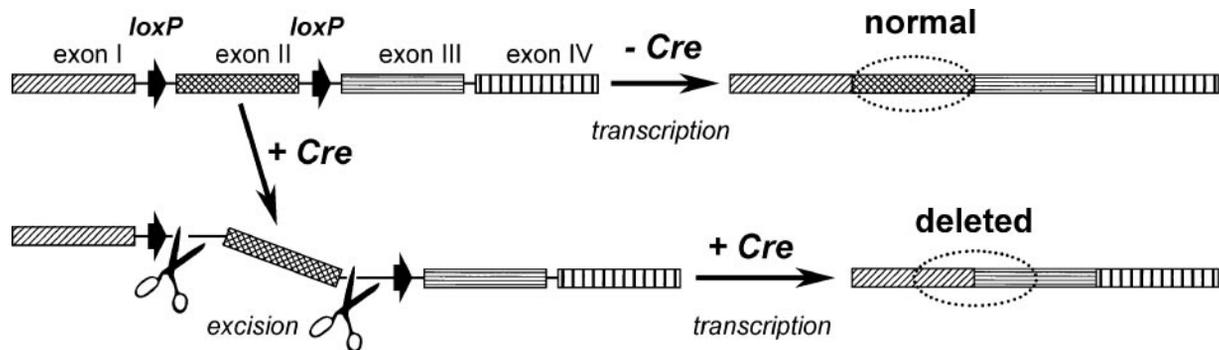


Fig. 3. Schematic of Cre-mediated gene deletion in a hypothetical gene. LoxP sites (arrowheads) are inserted into introns such that they flank critical coding or regulatory sequences, e.g., exon II. In the absence of Cre, normal gene transcription proceeds. In the presence of Cre, a recombination event results in excision of the loxP-flanked sequences, resulting in a dysfunctional transcript.

CA1- and pyramidal cell-restricted gene knockout using  $\alpha$ -CaMKII promoter-driven Cre transgenic mice. This study was important not only because it demonstrated the tissue/cell selectivity of the Cre-lox system, but it also provided important evidence that Cre-mediated gene excision can occur effectively in postmitotic cells such as neurons (117).

In brain regions for which there are no specific promoters available (e.g., sites where the brain RAS is expressed), recombinant viral vectors provide an important alternative for selective delivery of Cre. For example, Wang et al. (122) demonstrated Cre-mediated gene deletion in adult mouse brain using an Ad-encoded Cre vector. Stereotactically injected into specific regions within the cerebral cortex, hippocampus, and cerebellum, Ad-Cre-induced gene recombination was observed at high level in tissue at the injection sites (122). Gene deletion was also observed at lower levels in brain regions that send axonal projection to the injected regions (122), presumably due to retrograde transport of the Cre virus (see above).

We recently began to establish the tools and protocols necessary for using the Cre-lox system in combination with viral delivery of Cre to the brain to cell and site selectively delete genes of the RAS. For a number of reasons, we believe this will be a particularly powerful approach for teasing apart the intricacies of this system at the molecular, cellular, and functional levels. First, as detailed above, the regional and cellular specificity that can be achieved with Ad and FIV will be very useful for gene transfer of Cre to sites along the SFO-hypothalamic axis as well as others. Both site- and neuronal- or glial cell-restricted gene ablation should be possible with stereotaxic microinjection of Ad-Cre or FIV-Cre. Second, one of the important advantages of using virally delivered Cre is that the timing of the onset of gene ablation can be easily controlled. Although there are strategies for temporally restricting Cre expression using conditional Cre transgenic models (51), virally mediated Cre transfer is a straightforward approach for achieving similar goals, because this method can be employed at any time in the animal (within size limitations of the mouse). For example, we envision being able to use a powerful

“within-subjects” experimental design in which we are able to obtain baseline phenotype data before Cre injection and compare this to responses that occur within that same animal after Cre-mediated gene deletion. Finally, the viral vector approach to delivering Cre in a floxed model is more rapid and cost effective than maintaining an array of Cre transgenic lines.

To establish the feasibility of this approach for targeted ablation of the brain RAS, we employed a transgenic mouse model generated specifically as an *in vivo* “reporter” of Cre-mediated gene deletion (1; kind gift of Dr. M. Schneider). These mice harbor a transgene that consists of a loxP-flanked gene immediately upstream of the  $\beta$ -galactosidase gene. In the absence of Cre, the upstream gene prevents read-through expression of the  $\beta$ -gal gene. However, in the presence of Cre, the floxed gene is excised and expression of the reporter gene is permitted (1). Thus positive X-gal staining is an indicator of Cre-triggered gene excision. Using this reporter model in conjunction with stereotaxic injection of Ad-Cre or FIV-Cre, our recent preliminary studies indicate that highly localized Cre-mediated gene deletion can be achieved in a variety of nuclei along the SFO-hypothalamic axis (see below). An example of Ad-Cre-induced gene deletion in the SFO of this reporter model is shown in Fig. 4. Studies are ongoing to

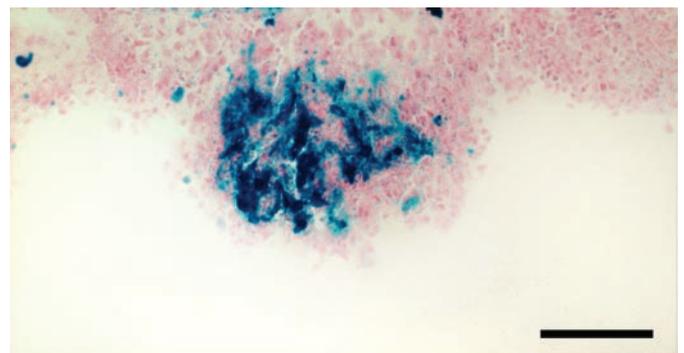


Fig. 4. Adenovirus-Cre-triggered gene deletion in the SFO of Cre reporter mouse. An adenovirus encoding Cre ( $1 \times 10^{12}$  particles/ml) was injected into the SFO of a mouse harboring a transgene that becomes activated only in the presence of Cre. Positive X-gal staining (blue) is an indication that gene deletion has occurred.

evaluate the efficiency, cell selectivity, and stability of Cre-mediated gene deletion in this brain circuitry. In parallel, development of transgenic models harboring floxed alleles of various RAS genes is also ongoing.

## CONCLUDING REMARKS

The ability to engineer the mouse genome has had a profound impact on biomedical research. The methodology has become increasingly sophisticated for achieving spatial, cellular, and temporal control over gene expression. The enormity of the potential for these experimental tools for increased understanding of normal physiology and the pathogenesis of disease cannot be overstated. The brain RAS is but one of many multifaceted systems for which application of these research strategies is ideal for unraveling the complex mechanisms by which it exerts its profound effects.

As powerful as these genomic strategies are, none of them are without limitations. For example, germline transgenesis has been known to result in unexpected developmental or physiological consequences that preclude carrying out the planned experiments. Embryonic lethal phenotypes in knockout models are the extreme example of this. Confounding compensatory alterations in gene expression or physiological systems as a result of lifelong transgenesis is also a potential limitation. Somatic gene transfer via viral vectors is also not without caveats. Despite advances in vector "backbones" to mutate or further delete viral genes, most currently available vectors contain residual viral sequences that can induce immunogenic responses. Gene transfer efficiency is also an issue that must be considered when using this approach. Technologies for both germline transgenesis and somatic gene transfer are rapidly advancing and improvements will undoubtedly be made. In addition, powerful new genomics tools such as RNA interference are beginning to emerge (126). However, even with the current technology, the numerous animal models already established, and the variety of reagents available, there is a wealth of information to be gained about the brain RAS and many other systems with appropriate experimental design, stringent controls, and careful interpretation of data.

Finally, although this review has focused on genomic strategies for dissecting the brain RAS, it would not be complete without some comments concerning phenotype assessment. The potential of these various gene manipulation strategies, as enormous as it is, can only be fully realized if the physiology used to determine the functional consequences is equally sophisticated. Indeed, advances in experimental approaches to assess cardiovascular function and fluid balance in mice have generally kept pace with the explosion in genetic tools. For example, methods for long-term evaluation of blood pressure, heart rate variability, and baroreflex control of heart rate in conscious freely moving mice are now available and have been employed in a number of physiological genomics experiments (21, 43, 48). The miniaturization of radiotelemetry for mice has been a key development in this regard (13). Imaging methods

such as ultrasound and MRI have also been important tools for cardiovascular phenotype assessment in mice (19, 123). Sophisticated assessment of water intake and urinary volume, electrolytes, and osmolality using metabolic cages is another important phenotyping tool (88). Although some parameters such as peripheral nerve activity are still challenging to evaluate in conscious mice, significant progress has been made in anesthetized preparations (68). As with genomic strategies, methods for integrative physiological analyses in mice will undoubtedly evolve.

With the combined use of state-of-the-art genomic strategies and physiological assessment, answers to long-sought questions about the brain RAS and many other complex cardiovascular regulatory systems are on the horizon.

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## DISCLOSURES

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