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# Sex chromosome complement involvement in angiotensin receptor sexual dimorphism

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ABSTRACT

This study aimed to define whether sex chromosome complement (SCC) may differentially modulate sex differences in relative gene expression of basal Agtr1a, Agtr2, and Mas1 receptors at fore/hindbrain nuclei and at medulla/cortical kidney.

Samples were collected from gonadectomized male (XX and XY) and female (XX and XY) mice of the "four core genotypes" model. At brain level, a SCC effect at the area postrema was demonstrated. An increase in mRNA level of Agtr1a and Agtr1a/Agtr2 ratio in XY-SCC mice was associated with a decrease in Mas1 compared to XX-SCC mice. In the renal cortex, a SCC effect for Agtr2 and Mas1 was observed. Regardless of sex (male or female), XX-SCC mice expressed higher levels of mRNA Agtr2 and Mas1 than XY-SCC mice {$F(1,12) = 6{,}126, p < 0.05$; $F(1,21) = 5{,}143, p < 0.05$}. Furthermore, XX-female mice showed a significant increase in Mas1 expression compared to XY-female mice.

These results reveal a SCC modulatory effect at central and kidney level on angiotensin receptor expression, with an enhancement of the vasodilatory arm in XX-mice and an increase in the vasoconstriction arm in XY-mice, which may underlie sex differences in the regulation of arterial pressure.



## 1. Introduction

Although awareness of sex differences in cardiovascular disease is increasing, much of what we know about blood pressure regulation has been derived from studies in males. However, principles learned in male models do not necessarily apply to females, and thus it is important to study the basis of sex differences.

The renin angiotensin system (RAS) exerts both hormonal and paracrine effects, modulating blood pressure regulation, among others. Clinical and basic findings demonstrate major sex differences in the way males and females respond to stimulation and inhibition of the RAS under physiological and pathophysiological circumstances (Brown et al., 2012; Sullivan, 2008; Xue et al., 2005) Differences in angiotensin peptides and receptors in males and females have been hypothesized to be one of the potential mechanisms contributing to sex-specific differences in cardiovascular homeostasis; which highlights the importance of studying basal brain and kidney RAS complements (Sandberg and Ji, 2012).

Ang II effects include a wide range of actions on the kidney, heart, blood vessels and adrenal gland in physiological and pathophysiological states. Furthermore, angiotensin peptides exert modulatory effects on the central nervous system, both in brain areas lacking the blood-brain-barrier (circulating Ang II-responders) as well as in brain areas in which Ang II is locally produced. Ang II binds to two G protein-coupled receptor (GPCR) subtypes, AT1 and AT2, exerting opposing and counterbalancing effects on the cardiovascular system. The classic excitatory effects evoked by Ang II (vasoconstriction, aldosterone and vasopressin release, sodium reabsorption, increased sympathetic activity and vascular growth) result from AT1 stimulation, whereas AT2 activation causes vasodilation, natriuresis, and anti-proliferation effects, thus opposing the vasoconstrictor and antinatriuretic effects of AT1-Ang II mediated responses (Carey et al., 2001; Li et al., 2003; Kaschina and Unger, 2003; Sandberg and Ji, 2000). Furthermore, Ang(1–7) mediates vasodilation via the AT2 receptor or its own receptor, the Mas receptor (Kaschina and Unger, 2003; Santos et al., 2003).

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Earlier studies showed differences in the ratio of AT1 to AT2 and Mas receptor expression between males and females (Sampson et al., 2012a,b; Silva-Antonialli et al., 2004), which may account for some of the Ang II-related sex differences associated with vasoconstrictor/vasodilator balance of the RAS. This leads to the question of what makes males and females different?

Exposure to sex steroids during critical periods of development can induce organizational (long-lasting or permanent) effects on sexually dimorphic traits. Sex steroids can also impart (temporary or reversible) activational effects at different times of life (during neonatal and peripubertal development as well as in adulthood) to cause most of the known sex differences in phenotype (Arnold and Gorski, 1984; McCarthy et al., 2012; Morris et al, 2004). For a long time, the organizational-activational dichotomy was applied to the understanding of many sex differences, and hormones were the only factors discussed as proximate signals causing sex differences. However, males and females differ not only in their sex (males are born with testes- and females with ovaries-hormonal factors) but also carry different sex chromosome complements (SCC: XY and XX respectively) and thus are influenced throughout life by different genomes. Exciting new data indicate that some genes escape X-inactivation and are expressed from both the "active" and "inactive" X chromosome, which may cause functional sex differences intrinsic to male (XY) and female (XX) cells, potentially contributing to sex differences in traits (sex-biased genes) (Carrel and Willard, 2005; Wolstenholme et al., 2013; Yang et al., 2006).

Although sex hormones (activational effects) are known to directly interact with RAS (Baiardi et al., 2005; Miller et al., 1999; Silbiger and Neugarten, 1995), the potential contribution of organizational hormonal and SCC effects on physiological sex-based difference in the regulation of the RAS remains undefined.

Taking into account that two of the components of the vasodilatador arm of the RAS (AT2 receptor (Agtr2) and ACE2 genes) are located in the X chromosome (Koike et al., 1994; Oudit et al., 2003) and that some genes escape X-inactivation (Carrel and Willard, 2005; Wolstenholme et al., 2013; Yang et al., 2006), it is tempting to speculate that genes residing in the SCC (which are asymmetrically inherited between males and females) may serve as candidate regulators of sexually dimorphic phenotypes. In this study, we sought to assess whether genetic differences within the SCC may differentially modulate the basal angiotensin type 1a (Agtr1a), 2 (Agtr2) and Mas (Mas1) receptor gene expression at kidney level and in brain nuclei involved in blood pressure regulation. We tested our hypothesis that the mRNA expression of the vasoconstrictor component (Agtr1a) of RAS would predominate in XY-SCC mice, while the vasodilator RAS components (Agtr2 and Mas1) would be enhanced in XX-SCC mice at specific brain nuclei and renal cortex levels.

To test our hypotheses, in the four core genotypes (FCG) mouse model, we evaluated relative mRNA expression levels of the angiotensin Agtr1a, Agtr2 and Mas1 receptors at different brain levels involved in blood pressure regulation and bradycardic baroreflex responses. We also evaluated angiotensinergic receptor expression in the renal cortex and medulla. The study was designed to evaluate the role of SCC and organizational hormonal effects, and therefore adult FCG mice were gonadectomized to remove the sex differences caused by the acute (activational) effects. Comparing gonadal males and females after gonadectomy can test whether having testes or ovaries causes long-lasting differences in the phenotype (organizational effect) while comparing mice with the same gonadal type but with different SCCs (XX versus XY) makes it possible to determine whether genes residing in the SCC differentially influence sexually dimorphic traits.

## 2. Methods

### 2.1. Animals

"Four core genotypes" mice were used in the following experiments. This mouse model combines a deletion of the testis-determining Sry gene from the Y chromosome ($Y^-$) and the subsequent insertion of a Sry transgene into an autosome. Sry gene deletion in XY mice ($XY^-$) yields a female phenotype (ovaries). When the Sry transgene is inserted into an autosome of these mice, they have testes and are fully fertile ($XY^-$Sry). The $Y^-$ chromosome and the Sry transgene segregate independently, and thus four types of offspring are produced by breeding $XY^-$Sry males with XX females: XX and $XY^-$ females (without Sry on the Y chromosome) and XX-Sry and $XY^-$Sry male mice (both with Sry in an autosome). All individuals possessing the Sry transgene develop testes and have a male external phenotype regardless of their SCC, while individuals lacking the transgene have ovaries and external female secondary sex characteristics. Male and female are defined here according to the gonadal phenotype. Throughout the text, we will refer to XX and $XY^-$ as XX and XY females, and to XXSry and $XY^-$Sry as XX and XY male mice respectively. By comparing these genotypes, it is possible to segregate the role of a) SCC (comparing mice with the same gonadal type but with different SCC: XX vs. XY) b) gonadal sex (males vs. females regardless of SCC), and c) the interaction of SCC and gonadal sex (Fig. 1).

MF1 transgenic mice, kindly provided by Dr. Paul Burgoyne from Medical Research Council National Institute for Medical Research, UK, were born and reared in the breeding facilities at the Instituto Ferreyra (Córdoba, Argentina). All experimental protocols were approved by the appropriate animal care and use committees at our institute, following the National Institutes of Health guidelines for the care and use of laboratory animals. Genotyping was performed as previously described in Caeiro et al., 2011.

### 2.2. Brain and kidney microdissection, tissue collection, RNA extraction and gene expression studies

Four core genotype mice (aged 45–50 days old) were gonadectomized to remove any activational effect of sex hormones that might mask the modulatory action of SCC and the organizational hormonal effects. After a 15-day-recovery period, the mice were decapitated and brain and kidneys were immediately excised and stored at −80 °C for Agtr1a, Agtr2 and Mas1 mRNA determination. Coronal sections of 590 μm (organum vasculosum laminae terminalis, OVLT), 720 μm (subfornical organ, SFO), 640 μm (paraventricular nucleus, PVN) and 560 μm (nucleus of the solitary tract, NTS and AP) were obtained from the frozen brains in a microtome with a stainless steel punch needle. The brain nuclei were identified and delimited according to the mouse brain atlas (Paxinos and Frank, 2001). Likewise, renal cortex and medulla samples were obtained. Longitudinal sections of 1600 μm were excised from the frozen kidney and punches were immediately taken with a stainless steel punch (inner diameter 1.5 mm).

RNA was isolated from punches of specific brain and kidney areas using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as directed by the manufacturer with some modifications: RNA precipitation with isopropanol was performed overnight at −20 °C. The RNA was treated with DNase (Fermentas) and quantified using a NanoDrop 2000 UV-Vis Spectrophotometer, and was then reverse-transcribed into cDNA (enzyme RTM-MLV – Promega). Brain Agtr1a and Agtr2 as well as brain and kidney Mas1 gene expression were determined using Sybr Green Real-Time PCR Master Mixes (Applied Biosystems™). Kidney Agtr1a and Agtr2 were determined with (Applied Biosystems™) in the Step One