Responses to Reviewers

Reviewer 1: Nihal Tumer

1. The Result section needs more detailed explanation.
Response-the Results section has been slightly expanded but the explanations of the main findings are expanded more in the Discussion section.

Reviewer 2: Toshio Kumai

1. Sry gene may not be activating tyrosine hydroxylase directly and what was the promoter gene was inserted in the vector?
Response- We agree, based largely on data published in our paper in Neuroscience Letters 369 (2004) 203-207. In that study we used the same Sry expression vector (which contains at CMV promoter) as used in the present study to transfect PC12 cells. A luciferase promoter gene under control of the tyrosine hydroxylase (Th) promoter was co-transfected with the Sry expression vector. Luciferase gene expression was increased, as a result of the Sry protein produced activating transcription from the Th promoter. When we used a Th promoter with a mutated AP-1 site, activation of Th by Sry was reduced by 75%, but not eliminated. These results indicate that Sry activates Th promoter activity, probably indirectly, at the AP-1 site in the Th promoter, as well as at other sites. Sry is a transcription factor that binds directly to the consensus sequence AACAATAGTAG (Harley et al. Nucleic Acids Res. 22 (1994) 1500-1501). The experiments in the Neurosci. Lett. paper did not allow us to determine whether Sry itself bound directly to the Th promoter DNA sequences or whether Sry activated another gene, whose product bound to Th promoter sequences to activate transcription.

2. Sry increased plasma NE but not E- better for Discussion
Response- we agree that more discussion is needed and have added the following to pg 7, 2nd para:
“Also, plasma E did not increase like NE did after Sry administration. An explanation for this may be that Sry is working on the enzyme, Th, and not on the enzyme that produces E, PNMT. Since both end products exist in the adrenal medulla there is not necessarily a 1/1 relationship with NE/E.

3. “Next …study…localization of Sry in adrenal”.
Response- We agree that this should be done, and we plan to do these experiments whenever we can obtain an antibody to rat Sry that detects Sry in tissues. At the present time, none is available.

Reviewer 3: John Imig

1. Strong association of Sry, Th and BP but no blocker experiments and no other systems examined to eliminate alternate or additional systems that may be involved.
Response- Our main point is that Sry increases BP and we have postulated one possible mechanism which is also supported by several of our publications showing the SHR Y chromosome increases several indices of SNS activity. By no means do we mean to exclude other systems that could be involved, such as the RAS, glucocorticoids and testosterone. Indeed, we have evidence that testosterone is involved in addition to Th. In preliminary experiments we delivered Th antisense to the kidney of WKY males and prevented the rise in BP that we observe after Sry delivery. However, since this paper deals with the adrenal gland we did not mention this. What we have done to address these issues is add further explanation to the Discussion- pg. 6, 2nd para-

“We provide evidence here that part of the BP rise may be due to Th in the adrenal medulla. This does not exclude other potential targets of Sry, such as, the renin-angiotensin system (RAS) and testosterone. We cannot eliminate the RAS as a player in the Sry story so further investigation is needed. However, we did examine plasma renin levels after Sry delivery to the kidney in WKY males and there was no renin difference from vector controls (unpublished data). With regards to the possibility that the adrenal cortex may have been involved due to the medullary injections we have not directly studied potential steroid targets of Sry, such as, adrenal aldosterone, glucocorticoids and testosterone. However, we have examined renal function and found normal sodium excretion and creatinine and albumin clearance (unpublished data). One would expect to see altered sodium excretion if Sry modulated plasma aldosterone levels. Although an indirect measure of a glucocorticoid effect, we have measured plasma and urinary glucose after Sry delivery to the kidney and found no effect compared to controls. Also testosterone most likely is an important factor promoting the BP rise since our previous studies have shown that a locus on the SHR Y chromosome produces an earlier rise in plasma testosterone compared to WKY males or SHR males with the WKY Y chromosome [23]. However, we have not yet made the connection between Sry and a direct rise in plasma testosterone. Sry could have an influence in hypothalamic and pituitary release of gonadotropins. This is an area for future study. A piece of evidence that would suggest that adrenal testosterone was not effected by adrenal medullary injection of Sry is that in castrated WKY males we do not detect any plasma testosterone even up to 10 weeks after castration which is enough time for adrenal compensation to take place [24]. Still it is possible that stringent stimulation of the medulla could release cortical testosterone. Indeed, a provocative area of research is the crosstalk between the adrenal cortex and medulla. A decrease in Th in chromaffin cells can decrease adrenal cortical function and conversely, an elevation in plasma catecholamines can elevate plasma aldosterone and glucocorticoids [25]. “

2. Were experiments done to determine the involvement of the SNS to the increase in BP?
Response- We only measured plasma and adrenal NE, E levels and Th as indices of SNS function. In our earlier work which we cited (refs.#2,4,5), we show that the locus on the SHR Y chromosome when bred into a WKY male produces increased NE turnover in heart and kidney compared to a normal WKY male. This study did not examine the adrenal turnover. Also we have added a study of ours (ref.23) that shows neonatal sympathectomy eliminated the SHR Y chromosome BP rise.
3. Does Sry injected into a female WKY have the same effect? This is a good question and one that we have recently examined for the kidney experiments which will be included in another manuscript. When Sry was delivered to the kidney of female WKY Th and plasma NE both increased 20% and BP increased. We have not examined female adrenal yet.

4. Figure 5 suggests Sry confined to the medulla.
   Response- due to ineffective Sry antibody from several sources we have not shown how far Sry diffuses or is transported. In preliminary experiments with GFP using the same procedure in the kidney we were able to show a small region effected close to the injection site (within a few glomeruli).

5. BP at all time points should be reported.
   Response- we agree and a new graph has been made with weekly BP- figure 1.

Reviewer 4: Maria Vieira-Coelho

1. a. Problem with hypothesis not being specific to tissue studied
   b. SHR have lower adrenal Th activity
   Response- a. We agree that the hypothesis needed to be directed to the adrenal medulla and was changed, pg 3, para 1 Background and in the abstract.
   b. We agree and had cited the reference by the reviewer that SHR had lower endogenous Th but higher plasma NE (old ref.#27, new ref# 34) as an example of how there is an uncoupling between Adrenal Th and release of plasma NE.

2. Improvement in Discussion needed: a. NE and E source issue
   b. adrenal cortical crosstalk issue
   Response- a. we agree that a better discussion was needed on the sources of the catecholamines and the plasma catechols levels. Added to Discussion, pg. 7, 2nd para- “. Also, plasma E did not increase like NE did after Sry administration. An explanation for this may be that the Sry protein is working on the enzyme, Th, and not on the enzyme that produces E, PNMT. Since both end products exist in the adrenal medulla there is not necessarily a 1/1 relationship with NE/E.”
   b. we agree and have added new refs and discussion of potential crosstalk, Discussion pg. 6, 2nd para-

“We provide evidence here that part of the BP rise may be due to Th in the adrenal medulla. This does not exclude other potential targets of Sry, such as, the renin-angiotensin system (RAS) and testosterone. We cannot eliminate the RAS as a player in the Sry story so further investigation is needed. However, we did examine plasma renin levels after Sry delivery to the kidney in WKY males and there was no renin difference from vector controls (unpublished data). With regards to the possibility that the adrenal cortex may have been involved due to the medullary injections we have not directly studied potential steroid targets of Sry, such as, adrenal aldosterone, glucocorticoids and testosterone. However, we have examined renal function and found normal sodium
excretion and creatinine and albumin clearance (unpublished data). One would expect to see altered sodium excretion if Sry modulated plasma aldosterone levels. Although an indirect measure of a glucocorticoid effect, we have measured plasma and urinary glucose after Sry delivery to the kidney and found no effect compared to controls. We have evidence that plasma corticosterone in WKY controls was not different than that in WKY males with the SHR Y chromosome [24]. This suggests that the SHR Y locus does not endogenously alter corticosterone, however, it is possible that Sry delivered to the adrenal medulla could have a corticosterone effect. Also testosterone most likely is an important factor promoting the BP rise since our previous studies have shown that a locus on the SHR Y chromosome produces an earlier rise in plasma testosterone compared to WKY males or SHR males with the WKY Y chromosome [25]. However, we have not yet made the connection between Sry and a direct rise in plasma testosterone. Sry could have an influence in hypothalamic and pituitary release of gonadotropins. This is an area for future study. A piece of evidence that would suggest that adrenal testosterone was not effected by adrenal medullary injection of Sry is that in castrated WKY males we do not detect any plasma testosterone even up to 10 weeks after castration which is enough time for adrenal compensation to take place [26]. Still it is possible that stringent stimulation of the medulla could release cortical testosterone.

Indeed, a provocative area of research is the crosstalk between the adrenal cortex and medulla. A decrease in Th in chromaffin cells can decrease adrenal cortical function and conversely, an elevation in plasma catecholamines can elevate plasma aldosterone and glucocorticoids [27].

3. Th method ref. And clarification- we agree that Nagatsu was the 1st and the others were slight modifications. This ref has been added as well as the L-Dopa formed per min and mg tissue to the text and figure.

4. Figure 2 control values for Th in relationship to Kumai’s values. Kumai used different units than we did and if his units are converted to ours for adrenal th in WKY he shows 16,670 fmol/mg/min and we show 37,494 for Sry and 25,181 fmol/mg/min for control values which is in the ballpark for each other. This has been added to the results for clarification- Results, pg. 5 1st para-

“Figure 2 shows that 3 weeks after Sry delivery to the adrenal medulla, Th was significantly increased compared to the vector control (37,494 vs. 25,181 fmol/min/mg, p=.017). These values were comparable to Kumai’s values in male WKY (16,670 fmol/mg/min converted to the units we used) [19].”

5. Minor points-

References were put in brackets
uM changed to µM
figure 4 dropped and data in text legends corrected
titles removed from figures