Evidence for a novel natriuretic peptide receptor that prefers brain natriuretic peptide over atrial natriuretic peptide

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Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) exert their physiological actions by binding to natriuretic peptide receptor A (NPRA), a receptor guanylate cyclase (rGC) that synthesizes cGMP in response to both ligands. The family of rGCs is rapidly expanding, and it is plausible that there might be additional, as yet undiscovered, rGCs whose function is to provide alternative signalling pathways for one or both of these peptides, particularly given the low affinity of NPRA for BNP. We have investigated this hypothesis, using a genetically modified (knockout) mouse in which the gene encoding NPRA has been disrupted. Enzyme assays and NPRA-specific Western blots performed on tissues from wild-type mice demonstrate that ANP-activated cGMP synthesis provides a good index of NPRA protein expression, which ranges from maximal in adrenal gland, lung, kidney, and testis to minimal in heart and colon. In contrast, immunoreactive NPRA is not detectable in tissues isolated from NPRA knockout animals and ANP- and BNP-stimulatable GC activities are markedly reduced in all mutant tissues. However, testis and adrenal gland retain statistically significant, high-affinity responses to BNP. This residual response to BNP cannot be accounted for by natriuretic peptide receptor B, or any other known mammalian rGC, suggesting the presence of a novel receptor in these tissues that prefers BNP over ANP.

Key words: adrenal gland, brain natriuretic peptide receptor, C-type natriuretic peptide, guanylate cyclase, testis.

INTRODUCTION

Natriuretic peptides (NPs) reduce blood pressure through integrated actions on a number of target organs [1–4]. Mice carrying mutations that disrupt the genes encoding atrial NP (ANP) or its receptor, NP receptor A [NPRA, also called guanylate cyclase (GC)-A], have higher than normal blood pressures and subsequent cardiovascular disease [5–7]. Mice that overexpress ANP or NPRA have, by contrast, lower than normal blood pressures [5,8,9]. This suggests that genetic polymorphisms in pathways that mediate NP signalling may contribute to hereditary hypertension, a disease that puts millions of humans at risk for stroke, heart disease and kidney failure.

The NP family includes three structurally related molecules. ANP, the first identified member of the family, is produced primarily by atrial myocytes, where it is stored in granules and released when the myocytes are subjected to mechanical stretch [2]. Once in the circulation, ANP triggers a variety of mechanisms that reduce blood pressure. For example, it induces natriuretic and diuretic responses in the kidney, reduces total peripheral resistance, inhibits the activity of the renin–angiotensin–aldosterone system, and suppresses the output of the sympathetic nervous system [2,3]. These ANP-activated responses are primarily mediated by NPRA [1], a receptor GC (rGC) that synthesizes cGMP when exposed to ANP [10,11]. The elevated cGMP in turn activates downstream effectors, such as cGMP-dependent protein kinases [12], cGMP-gated channels [13] and cGMP-sensitive phosphodiesterases [14], in order to bring about the physiological responses.

The second member of the NP family, B-type NP (BNP), was first detected in extracts of porcine brain [15], but was subsequently shown, like ANP, to be expressed primarily in the myocardium [16]. Also like ANP, BNP is released into the circulation when the heart is stretched [17], and its known physiological effects on target organs are similar to those of ANP. However, in contrast to ANP, which is almost exclusively found in atria, a significant amount of BNP is present in ventricular myocytes [18], suggesting that in some circumstances the two peptides might play distinct physiological roles.

A third NP, C-type NP (CNP), is expressed primarily in non-cardiac tissues [19] and is found only at low or undetectable levels in plasma [20,21]. Its actions are thought to be paracrine rather than endocrine in nature [4]. CNP also differs from ANP and BNP in that it has little intrinsic natriuretic activity [22], though it does play an important role in cardiovascular physiology as a potent vasorelaxant [4], as well as an inhibitor of vascular smooth muscle proliferation [23] and endothelial cell migration [24].

As might be expected from its distinct spectrum of physiological actions, CNP does not act through NPRA, the receptor that mediates the effects of ANP. Instead, CNP selectively activates NP receptor B [NPRB (also referred to as GC-B)], a second rGC that is related to NPRA, but only weakly sensitive to ANP and BNP [25]. Surprisingly, the identity of the receptor that mediates the effects of BNP is not well established. BNP can activate NPRA, though its potency is about 10-fold lower than that of ANP [26,27], and at least some of the physiological actions of BNP have been shown to depend on the presence of functional NPRA [28]. However, the relatively low affinity of NPRA for BNP has led to speculation that an additional BNP-

Abbreviations used: NP, natriuretic peptide; ANP, atrial NP; BNP, B-type NP; CNP, C-type NP; GC, guanylate cyclase; IBMX, 3-isobutyl-1-methylxanthine; NPRA, NP receptor A; NPRB, NP receptor B; NPRC, NP receptor C; *Npr1*, the gene encoding NPRA in mice; rGC, receptor GC.

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specific rGC remains to be discovered [29]. Indeed, the rapid growth of the rGC family in recent years [30] leaves open the possibility that there might be additional rGCs that mediate responses to ANP as well, analogous to the multi-component receptor families that are targeted by other hormones and neurotransmitters.

It is difficult to establish the existence of additional BNP- or ANP-sensitive rGCs against the background of prominent NPRA expression present in a normal animal. However, gene targeting has recently been used to produce knockout mice completely lacking NPRA [7,31], providing an ideal system in which to examine this possibility. In the present study we show that NPRA-associated immunoreactivity and NPRA-associated enzyme activity can be detected in a variety of wild-type tissues, whereas in knockout animals immunoreactive NPRA can no longer be detected and responses to both ANP and BNP are greatly attenuated. However, *Npr1*$^{-/-}$ (*Npr1* is the gene encoding NPRA in mice) animals retain a statistically significant level of residual, BNP-preferring enzyme activity in testis and adrenal gland, suggesting the presence of a novel, BNP-selective receptor in these tissues.

EXPERIMENTAL

Mouse genotype analysis

The wild-type (*Npr1*$^{+/+}$) and knockout (*Npr1*$^{-/-}$) mice used in this study were of a mixed 129/C57BL6 genetic background derived from the original mutants reported previously [7]. Animals were genotyped by multiplex PCR using: primer A (5′-GCTCT-CTTGTCGCCGAATCT-3′), a sequence 5′ to the mouse *Npr1* gene common to both alleles; primer B (5′-TTTAGAGCA-GGTGAGAGCGA-3′), an exon 1 sequence only present in the intact mouse allele; and primer C (5′-GCTTCCTCGTGCT-TTACGGT-3′), a sequence in the neomycin resistance cassette only present in the null allele. The PCR reaction from tail DNA included 50 mM Tris, 20 mM ammonium sulphate, 1.5 mM $MgCl_2$, 10 % DMSO, 100 μM of each dNTP, 2 units of *Taq* polymerase and 40 nM primers. The PCR cycling conditions were 30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C for 35 cycles. PCR products were resolved on a 2 % agarose gel. Amplified products corresponding to the wild-type and targeted alleles were 330 bp and 250 bp respectively.

GC assays

Adrenal gland, lung, kidney, testis, liver, colon, forebrain, heart, and thoracic aorta were removed from 2–6-month-old mice euthanized by anaesthetic overdose with avertin or urethane. For adrenal gland and aorta, tissues were pooled from multiple animals in order to obtain sufficient material. After dissection, tissues were rinsed with Buffer B (50 mM Hepes, 1 mM EDTA and 0.01 % bacitracin) and homogenized in ice cold Buffer B (1 ml/0.5 g of tissue weight) using a motor-driven Potter–Elvehjem homogenizer with a glass pestle. After an initial centrifugation at 800 ***g*** for 5 min at 4 °C to remove tissue fragments, the supernatant fraction was centrifuged at 100 000 ***g*** for 1 h at 4 °C in a Ti-50 rotor (Beckman Instruments). The high speed supernatant fraction was then discarded, and the pellet was resuspended by homogenizing in ice cold Buffer B (1 ml/0.5 g of original tissue weight), diluted 8-fold with Buffer B, and stored on ice until assayed for activity, usually within 15 min. Protein content was determined on a portion of the diluted membranes (solubilized in 1 M NaOH) using a dye-binding assay (Bradford assay, BioRad).

To initiate cyclase activity, a portion of the diluted membranes was incubated with an equal volume of Buffer A [50 mM Hepes, 8 mM $MgCl_2$, 2 mM 3-isobutyl-1-methylxanthine (IBMX), 4 mM ATP, 4 mM GTP, 60 mM phosphocreatine, 800 μg/ml creatine phosphokinase (185 units/mg) and 1 mg/ml BSA] at 37 °C, with or without ANP-28, BNP-45, or CNP-22 [all peptides were the rat or mouse form (Peninsula or Phoenix Pharmaceuticals)]. At various times thereafter, 40 μl samples were pipetted from this incubation mixture into 100 μl of 6 % (w/v) trichloroacetic acid. Each sample was then extracted four times with 3 volumes of diethyl ether, to remove trichloroacetic acid, and the cGMP content was determined by radio-immunoassay. Results are expressed as pmol of cGMP synthesized/mg of protein per min, as determined by linear regression analysis of 0, 5 and 10 min time points. Basal and ligand-stimulated rates of cGMP synthesis are always measured in parallel for each membrane preparation. Net ligand-stimulated cGMP synthesis is represented by the difference between these two rates. To avoid problems that could arise due to sequestration of ligands by NPRC (a clearance receptor that binds all known NPs with high capacity), we typically used ANP, BNP and CNP at 0.1 or 1 μM. This is well above the concentrations required to saturate NPRC, which range from 0.01–1 nM, depending on the ligand, the source of receptor and the assay conditions [32].

Every individual determination represents results obtained from an independent animal. To eliminate assay-to-assay variability, the elements of each data set were always analysed in a single immuno-assay. To facilitate interassay comparisons, all data sets have been normalized so that the basal rates of cGMP synthesis measured in lung membranes are distributed about a common mean value (chosen arbitrarily as the basal rate obtained from our largest data set). Statistically significant differences between treatment groups ($P < 0.05$) were confirmed using either an unpaired or, where appropriate, a paired two-tailed *t*-test (Microsoft Excel). For concentration–response curves (see Figures 3 and 8), results were fitted either with a two-site model:

$$SR/CR = 1 + [(V_{max}^{HA} \cdot np)/(EC_{50}^{HA} + np)] + [(V_{max}^{LA} \cdot np)/(EC_{50}^{HA} + np)]$$

where SR is the stimulated rate of cGMP synthesis (cGMP synthesized/mg of protein per min), CR is the control rate (cGMP synthesized/mg of protein per min), *np* is the concentration (in M) of the natriuretic peptide used (ANP, BNP or CNP), V_{max}^{HA} and EC_{50}^{HA} are the V_{max} and EC_{50} of the high affinity site, V_{max}^{LA} and EC_{50}^{LA} are the V_{max} and EC_{50} of the low affinity site, or a one-site model (in which V_{max}^{LA} is zero).

Western blotting

Organs were obtained as above, and homogenized with a polytron (Brinkmann, Westbury, NY, U.S.A.) in ice-cold buffer C [20 mM Tris(hydroxymethyl)-2-aminoethanesulphonic acid, 10 mM mannitol, pH 7.4, 30 μg/ml PMSF, 2 μg/ml leupeptin and 16 μg/ml benzamidine]. Membrane and cytoplasmic fractions were separated by centrifugation, as described above, and membranes were resuspended in buffer C containing 1 % (v/v) Triton X-100, then re-centrifuged to yield Triton X-100-soluble and -insoluble fractions. Protein concentrations were measured using the bicinchoninic acid protein assay reagent (Pierce).

Protein (60 μg) in the Triton X-100-soluble fraction was electrophoresed on SDS/PAGE (7.5 % gels), transferred to Immobilon-P and analysed by chemiluminescent Western blotting as described previously [7] using antiserum 281. This antibody is directed against the rat NPRA extracellular domain, and was generated by immunizing rabbits with recombinant

polyhistidine-tagged NPRA [residues 242–493] purified after bacterial expression (P. J. Mohler and S. L. Milgram, unpublished work). Immunoreactive bands corresponding to NPRA are found only in the Triton X-100-soluble fraction.

RESULTS

Evaluation of NPRA expression by Western blotting

We performed immunoblots with an NPRA-specific polyclonal antibody as an activity-independent way to assess the relative abundance of NPRA in various tissues. In *Npr1*$^{+/+}$ tissues, NPRA typically appears as a broad heterogeneous zone of immunoreactivity whose apparent molecular mass (spanning 130–135 kDa, marked by the arrow in Figure 1a) agrees with previous estimates [10,33]. A tissue comparison (Figure 1b) reveals that the specific activity of this immunoreactive material is high in *Npr1*$^{+/+}$ lung, kidney, adrenal gland, liver and testis, while expression is below the limit of detection in *Npr1*$^{+/+}$ colon, brain, heart and aorta. No NPRA-associated immunoreactivity is observed in any *Npr1*$^{-/-}$ tissue.

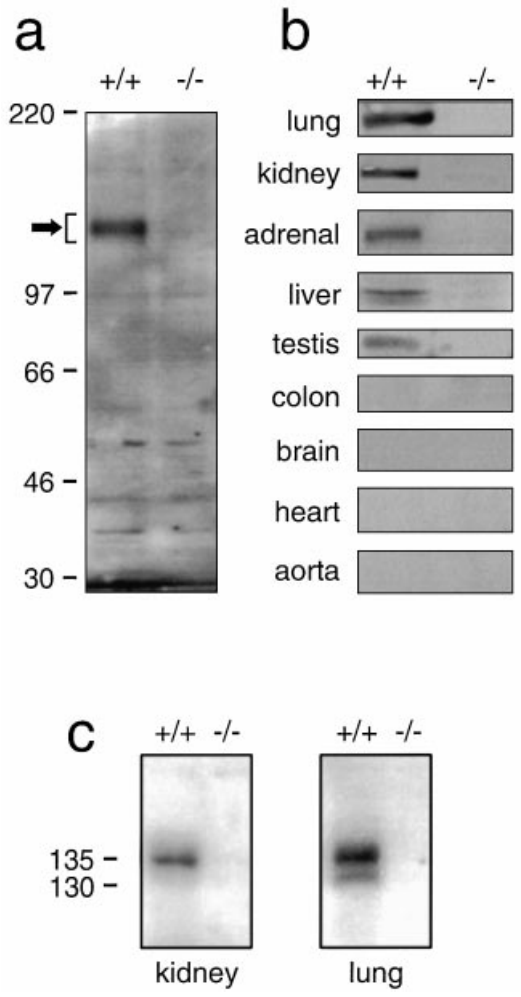

Figure 1 Immunoblot analysis of NPRA expression

Western blots were performed on tissue extracts from wild-type (+/+) or NPRA knockout (−/−) mice, as indicated, using a polyclonal antibody raised against an isoform-specific region of the extracellular domain of rat NPRA. Each lane of a 7.5% PAGE was loaded with 60 μg of membrane protein. (**a**) A full-length immunoblot shows a broad band of immunoreactivity in the 130–135 kDa range (marked by the arrow) which, based on size and genotype-dependent expression levels, corresponds to NPRA. (**b**) Relevant regions (130–135 kDa) taken from immunoblots performed on the indicated panel of tissues. Adjacent lanes were loaded with equivalent amounts of protein extracted from *Npr1*$^{+/+}$ and *Npr1*$^{-/-}$ membranes. (**c**) Comparison of NPRA from lung and kidney on a gel that fortuitously displayed exceptional resolution, revealing differential processing of NPRA in the two tissues.

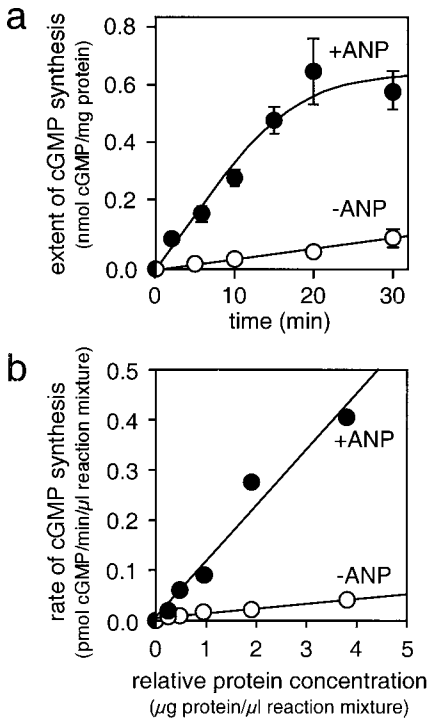

Figure 2 Characterization of the GC assay

(**a**) A time course of cGMP synthesis was established for *Npr1*$^{+/+}$ kidney membranes in the presence (●) or absence (○) of 1 μM ANP over a 30-min period. Each symbol represents the mean value determined for membrane samples from two independent animals, each assayed in duplicate. Error bars indicate the range defined by the two animals. (**b**) Cyclase activity is proportional to the amount of membranes included in the assay. Each point indicates the initial rate of cGMP synthesis (i.e. the slope of a line fit by linear regression to duplicate 0, 5 and 10 min time points) in the presence (●) or absence (○) of 1 μM ANP, plotted as a function of the protein concentration of the extract. Results are representative of four similar experiments. In all cases, cyclase activity was linear over the range of protein concentrations used in our studies.

The complex and somewhat variable appearance of the NPRA band is likely due to the presence of alternatively processed subspecies with different degrees of N-linked glycosylation, as has been demonstrated previously in cell lines that overexpress recombinant NPRA [34,35]. Our results suggest that processing of native NPRA *in vivo* may occur in a tissue-specific manner. In adjacent lanes on gels with fortuitously high resolution, NPRA from *Npr1*$^{+/+}$ kidney migrates as a distinct single band of approx. 135 kDa, whereas NPRA from *Npr1*$^{+/+}$ lung migrates as a doublet of approx. 130 and 135 kDa (Figure 1c).

Evaluation of NPRA and NPRB expression by enzymic assay

We have used a quantitative cyclase assay to evaluate rGC-associated enzyme activity. The assay measures the rate at which cGMP is synthesized by isolated membranes in the presence and absence of ligand under standard optimized conditions. Our initial studies investigated the linearity of the cyclase assay with respect to time and protein concentration. The time course of basal and ANP-activated cGMP synthesis is illustrated in Figure

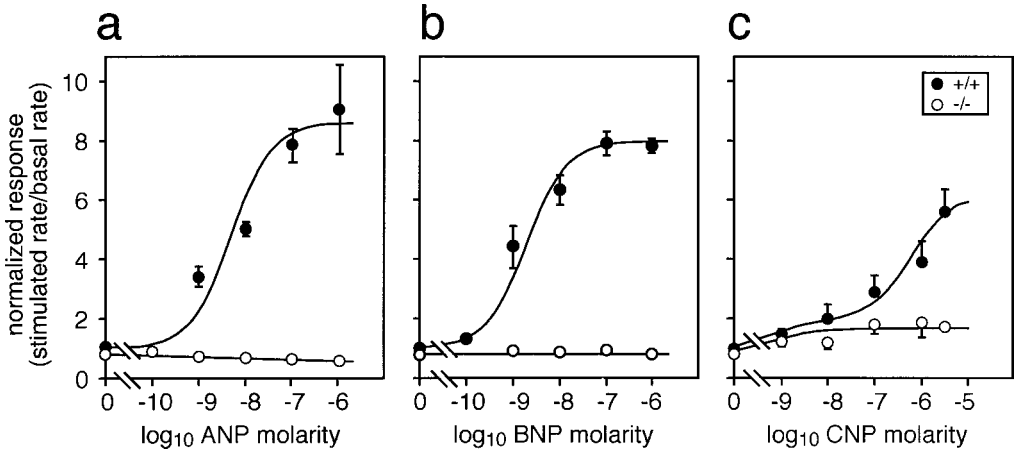

Figure 3 Sensitivity of lung membranes to ANP, BNP and CNP

Concentration–response analysis of the effects of ligands on lung membranes from *Npr1*$^{-/-}$ (○) or *Npr1*$^{+/+}$ (●) animals. All results are reported as fold-stimulation (stimulated rate/basal rate). The results for *Npr1*$^{-/-}$ animals have been adjusted to reflect the fact that the basal rate in *Npr1*$^{-/-}$ lung is 79% of the basal rate in *Npr1*$^{+/+}$ lung (see Figure 9). In (**a**) each point provides the mean value determined for two independent animals, and the error bars indicate the range. In (**b**) each *Npr1*$^{+/+}$ point reports the mean ± S.E.M. ($n = 4$), whereas each *Npr1*$^{-/-}$ point provides the mean ± range ($n = 2$). For (**c**), each point gives the mean ± S.E.M. ($n = 3$). Solid lines were calculated using either a one- or a two-site model, as described in the Experimental section. The EC_{50} used to calculate the *Npr1*$^{+/+}$ ANP responses was 5 nM; V_{max} for *Npr1*$^{+/+}$ ANP responses was 7.6; EC_{50} for *Npr1*$^{+/+}$ BNP responses was 2 nM; V_{max} for *Npr1*$^{+/+}$ BNP responses was 7.0; EC_{50}(low affinity) for *Npr1*$^{+/+}$ CNP responses was 0.6 μM; V_{max}(low affinity) for *Npr1*$^{+/+}$ CNP responses was 4.3; EC_{50}(high affinity) for *Npr1*$^{+/+}$ CNP responses was 0.6 nM; V_{max}(high affinity) for *Npr1*$^{+/+}$ CNP responses was 1; EC_{50}(high affinity) for *Npr1*$^{-/-}$ CNP responses was 0.6 nM; and V_{max}(high affinity) for *Npr1*$^{-/-}$ CNP responses was 0.9.

2(a) for *Npr1*$^{+/+}$ kidney membranes. In the absence of agonist the reaction is linear over 30 min, while in the presence of agonist the rate is approximately linear over the first 10 min and declines to near zero by 30 min. Non-linear reaction kinetics have been observed previously for agonist-activated NPRA, and are thought to reflect receptor desensitization [36]. Despite this inactivation process, the initial linear rate of cGMP synthesis in the presence of agonist can be quantified by regression analysis of 0, 5 and 10 minute time points. For the time course shown in Figure 2a, the initial rate under basal conditions is 2.4 pmol/min per mg of protein, while in the presence of ANP it is 34 pmol/min per mg of protein (nearly a 14-fold increase).

The assay is also linear over a wide range of input activity (Figure 2b), and the constant ratio between stimulated and control rates indicates that the cyclase assay should accurately reflect relative enzyme levels despite differences in protein yield from preparation to preparation. We routinely use protein concentrations of 1.25 μg/μl or less, well within the lower end of the range of concentrations tested here.

We then used membranes from lung, a tissue that expresses relatively high levels of both NPRA and NPRB mRNA [26,37], to characterize the properties of the murine forms of these rGCs in a native setting. As shown in Figure 3(a), the EC_{50} for ANP in *Npr1*$^{+/+}$ membranes is approx. 5 nM, in good agreement with previous reports [26,27]. In contrast, *Npr1*$^{-/-}$ membranes are quite insensitive to ANP, even at the highest concentration tested. The EC_{50} for BNP in *Npr1*$^{+/+}$ membranes is 2 nM (Figure 3b), again in reasonable agreement with previous reports [26,27], although surprisingly we failed to observe a rightward shift in the potency of BNP relative to ANP. This may reflect the fact that we were comparing mouse BNP-45 provided by one manufacturer to rat ANP-28 provided by a different manufacturer. As with ANP, lung membranes from *Npr1*$^{-/-}$ animals are essentially insensitive to BNP. When tested with CNP, membranes from *Npr1*$^{+/+}$ lung clearly show both high and low affinity responses (Figure 3c). The EC_{50} of the high affinity component is approx. 0.6 nM, consistent with an NPRB-mediated response [27,38], whereas the EC_{50} for the low affinity component is well above 100 nM. It is likely that the low affinity response is a consequence of cross-activation of NPRA by CNP, since this component is absent in membranes obtained from the N*pr1*$^{-/-}$ animals (Figure 3c). This idea is compatible with the known low affinity of recombinant NPRA for CNP [27].

These results give rise to several interesting observations. First, the lack of effect of ANP and BNP in *Npr1*$^{-/-}$ membranes indicates that the mutation is not 'leaky', and that NPRA is the only rGC that mediates responses to both of these peptides in lung. Most importantly, ANP and BNP do not significantly cross-activate NPRB, which is clearly present in *Npr1*$^{-/-}$ lung membranes. Second, the maximal activity of NPRB in lung is much less than the maximal activity of NPRA. Third, the activity of NPRB has not been significantly up- or down-regulated in *Npr1*$^{-/-}$ animals, indicating a lack of compensatory regulation following the ablation of NPRA. This can be seen by focusing on the appropriate portions of the concentration–response curves in Figure 3c (over the range of 0–10 nM ligand), where it is apparent, despite some scatter, that the magnitudes of the high affinity CNP responses are quite similar in both *Npr1*$^{+/+}$ and *Npr1*$^{-/-}$ animals.

Tissue survey of ANP-dependent rGC activity

Although the results in Figure 3 demonstrate that NPRA is the only rGC in lung that responds to ANP or BNP, it is possible that alternative rGCs might be expressed in other tissues. Figure 4 shows basal and stimulated rates of membrane cyclase activity in a number of *Npr1*$^{+/+}$ and *Npr1*$^{-/-}$ tissues tested with a saturating dose of ANP. In *Npr1*$^{+/+}$ animals, adrenal gland, kidney, lung and testis show the greatest responses to ANP, while liver and brain display small responses. Heart and colon respond only marginally. Surprisingly, thoracic aorta has no detectable cyclase activity, even in the presence of ANP, although the amount of protein included in the assay was comparable to that tested for other tissues.

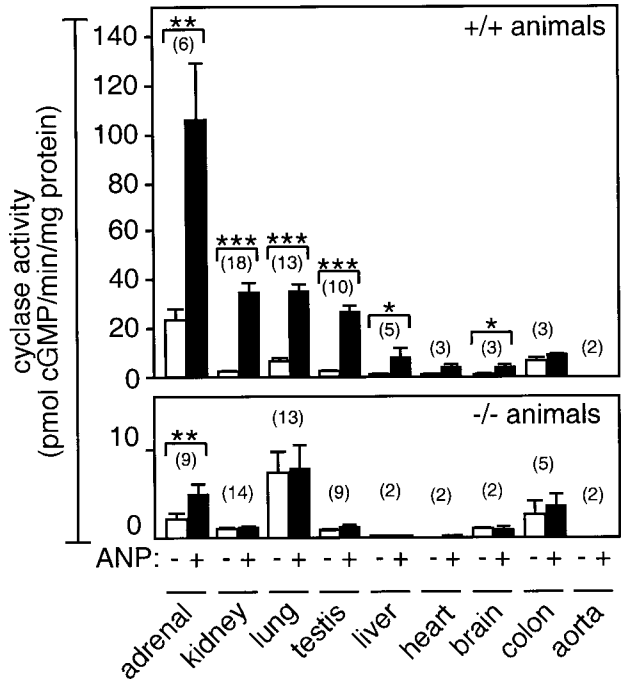

Figure 4 Tissue distribution of ANP-stimulated GC activity

Initial rates of cGMP synthesis were determined for plasma membranes isolated from organs of *Npr1*$^{+/+}$ mice (upper panel), or *Npr1*$^{-/-}$ mice (lower panel) in the presence (+) or absence (−) of a saturating dose of ANP (mean ± S.E.M. for responses to 1 μM agonist). Note the difference in scales between the upper and lower panels. The numbers in parentheses denote the number of independent animals tested. *, $P < 0.05$ relative to control; **, $P < 0.01$; ***, $P < 0.001$.

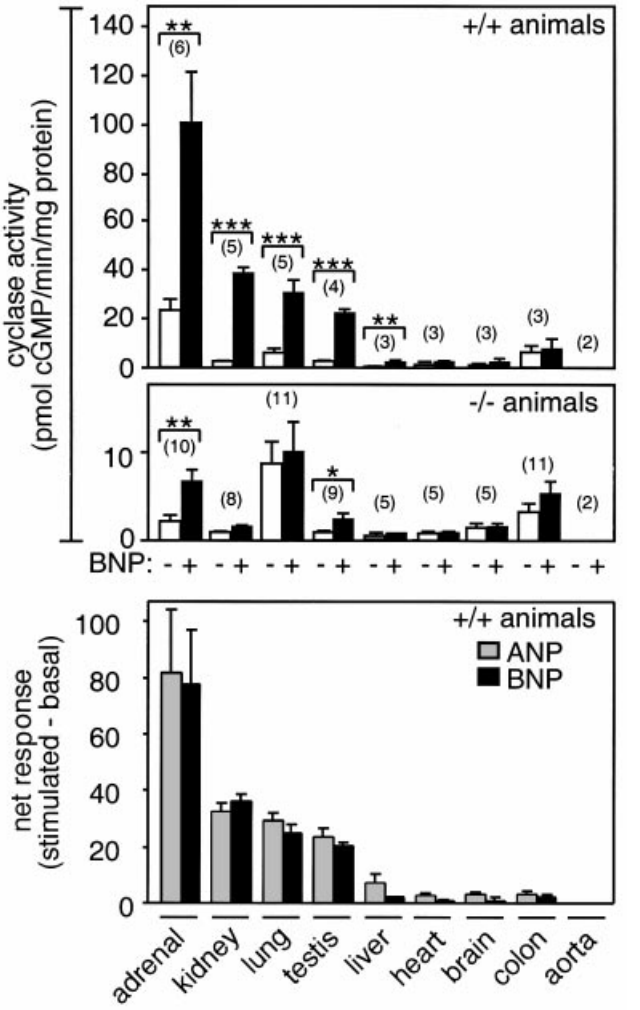

Figure 5 Tissue distribution of BNP-stimulated GC activity

Initial rates of cGMP synthesis were determined for plasma membranes isolated from organs of *Npr1*$^{+/+}$ mice (upper panel), or *Npr1*$^{-/-}$ mice (middle panel) in the presence (+) or absence (−) of a saturating dose of BNP (mean ± S.E.M. for pooled responses to 0.1 and 1 μM of agonist). Note the difference in scales between the upper and middle panels. The numbers in parentheses denote the number of independent animals tested. *, $P < 0.05$ relative to control; **, $P < 0.01$; ***, $P < 0.001$. The bottom panel shows each *NPRA*$^{+/+}$ tissue's average net BNP response (black bars) plotted adjacent to that same tissue's average net ANP response (grey bars). The net response is determined for each sample of membranes tested by subtracting its basal rate from its stimulated rate.

In contrast, *Npr1*$^{-/-}$ tissues are almost completely unresponsive to ANP (although, as described below, small residual responses are observed in adrenal gland). This argues that, in the tissues surveyed, ANP at 10 μM does not significantly activate any rGC other than NPRA. Therefore, the magnitudes of the responses to ANP observed in *Npr1*$^{+/+}$ tissues should provide an accurate index of NPRA expression. Indeed, the distribution of the biochemical responses correlates well with the distribution of NPRA immunoreactivity, shown in Figure 1(b), though the enzyme assay is obviously more sensitive than Western blotting.

NPRA is the dominant GC receptor for BNP

We next measured cGMP synthesis by membranes isolated from various wild-type tissues under control conditions or in response to a saturating concentration of BNP (Figure 5, upper panel). Adrenal gland, kidney, lung and testis exhibit robust responses, liver displays a small response, heart, brain and colon respond marginally, if at all, and activity is again undetectable in aorta. The pattern of sensitivity to BNP is similar to the pattern established for ANP (Figure 4, upper panel), and there is good agreement when the relative magnitudes of the BNP responses (stimulated rate minus basal rate) are compared to the relative magnitudes of the ANP responses determined in the same tissue (Figure 5, lower panel). These results indicate that NPRA is the dominant rGC responsible for BNP-activated cGMP synthesis, at least when high concentrations of BNP are applied.

We then examined responses to BNP in *Npr1*$^{-/-}$ mice, where the elimination of NPRA makes it easier to detect the activity of any additional BNP-sensitive GC (Figure 5, middle panel). The effects of the peptide are markedly attenuated in all tissues, again confirming that NPRA is the most abundant rGC capable of synthesizing cGMP in response to BNP. However, as with ANP, small but statistically significant responses are retained by some of the *Npr1*$^{-/-}$ tissues, most prominently testis and adrenal gland. Indeed, in *Npr1*$^{-/-}$ animals the effects of BNP on these two tissues are noticeably greater than those provoked by ANP, and amount to about 10 % of the BNP responses measured in the corresponding *Npr1*$^{+/+}$ tissues (Figure 6).

Residual BNP responses in *Npr1*$^{-/-}$ animals are not mediated by NPRB

At high concentrations, both ANP and BNP can activate the GC activity of rat NPRB [26,27,38]. Thus, if particularly high levels

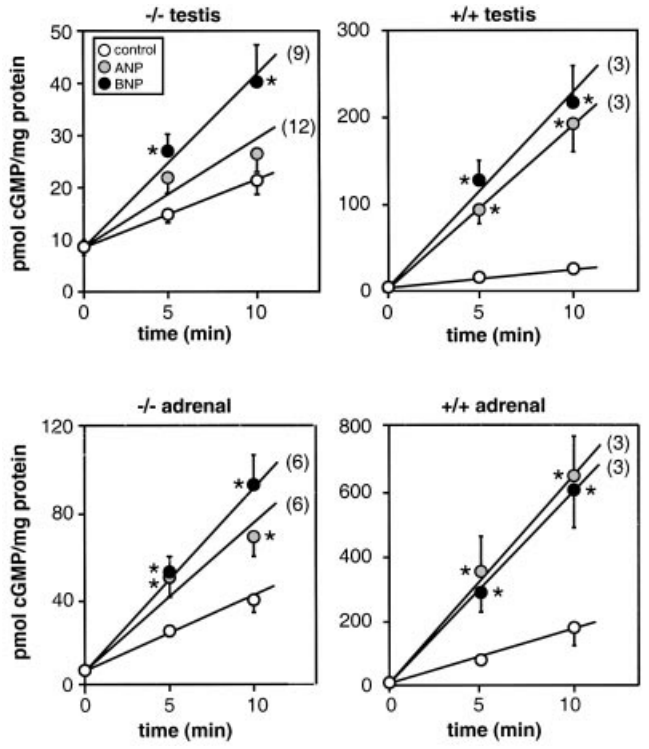

Figure 6 Time courses of basal and agonist-activated cGMP synthesis carried out by membranes from *Npr1*$^{-/-}$ and *Npr1*$^{+/+}$ testis and adrenal gland

Each panel shows the effects of ANP and BNP on the initial rate of cGMP synthesis by *Npr1*$^{-/-}$ membranes (left panels) or *Npr1*$^{+/+}$ membranes (right panels), analysed over a 10 min time course. The points report the mean ± S.E.M. at each time point for membranes stimulated with 1 μM ANP or 1 μM BNP, and their respective paired controls. The numbers in parentheses denote the numbers of animals tested with each ligand. Note the differences in scale between the *Npr1*$^{-/-}$ and *Npr1*$^{+/+}$ tissues. *, $P < 0.05$ relative to control.

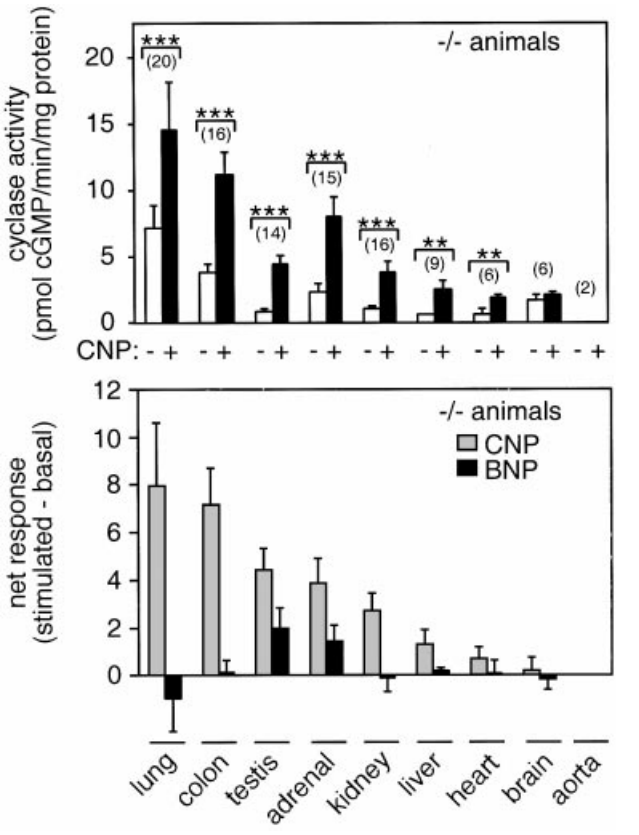

Figure 7 Tissue distribution of CNP-stimulated GC activity in *Npr1*$^{-/-}$ mice

The upper panel shows initial rates of cGMP synthesis determined for plasma membranes isolated from organs of *Npr1*$^{-/-}$ mice in the presence (+) or absence (−) of a saturating dose of CNP (mean ± S.E.M. for pooled responses to 0.1 and 1 μM of agonist). The number in parentheses denotes the number of independent animals tested. *, $P < 0.05$ relative to control; **, $P < 0.01$; ***, $P < 0.001$. The lower panel compares each mutant tissue's net CNP response (stimulated rate minus basal rate, grey bars) to that same tissue's net response to 0.1 μM BNP (stimulated rate minus basal rate, filled bars).

of NPRB were to be expressed in *Npr1*$^{-/-}$ testis and adrenal gland, then they could account for the residual responses to BNP and ANP. NPRB levels can be quantitated in an *Npr1*$^{-/-}$ tissue by measuring the tissue's response to a saturating dose of CNP, since we have shown that CNP becomes a selective NPRB agonist in the absence of NPRA (Figure 3c). Figure 7 (upper panel) shows that testis and adrenal gland do not express unusually high levels of NPRB relative to other tissues. Furthermore, the tissue distribution of the net responses (stimulated rate minus basal rate) elicited by CNP in *Npr1*$^{-/-}$ animals is distinct from the distribution of the net responses elicited by BNP (Figure 7, lower panel). For example, both *Npr1*$^{-/-}$ colon and lung respond robustly to CNP, but are completely insensitive to BNP, whereas *Npr1*$^{-/-}$ testis and adrenal gland provide the largest responses to BNP and are only moderately sensitive to CNP. This demonstrates that BNP and CNP cannot both be acting through the same receptor (i.e. NPRB), since if they were, the ratio of the two responses should remain constant in every tissue.

To further investigate the residual responses present in *Npr1*$^{-/-}$ testis, we evaluated the effects of a full range of agonist concentrations (Figure 8). The threshold for the BNP response is in the vicinity of 1 nM, while the threshold for the ANP response is approx. 100-fold higher. We obtained a good fit to the BNP results by using a single-site model with an EC_{50} of 20 nM,

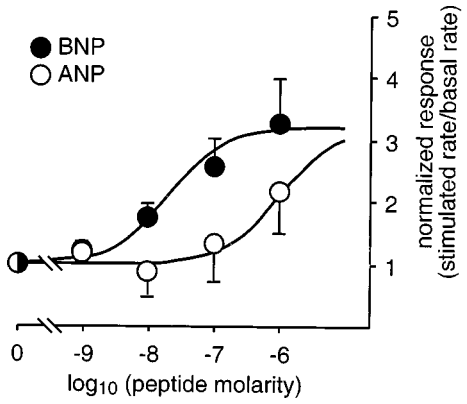

Figure 8 Testis membranes from *Npr1*$^{-/-}$ animals respond to BNP with high affinity

Analysis of the effects of ANP (○) or BNP (●) on cGMP synthesis by testis membranes (stimulated rate/basal rate). Each point gives the mean ± S.E.M. ($n = 7$ for BNP and $n = 5$ for ANP). The curves were calculated using a single-binding-site equation, as described in the Experimental section. Values for V_{max} (BNP, 2.2; ANP, 2.2) and EC_{50} (BNP, 2 nM; ANP, 1 μM) were adjusted by eye to provide an optimal fit.

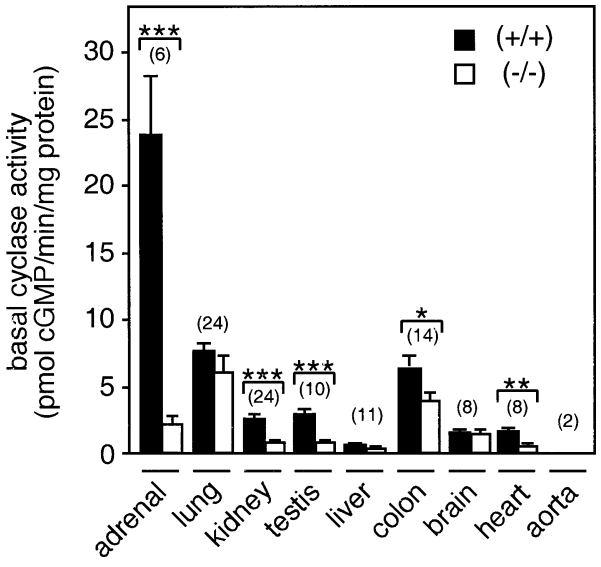

Figure 9 Basal GC activity varies from tissue-to-tissue, and reflects variable contributions of NPRA

Initial basal rates of cGMP synthesis were determined for plasma membranes isolated from organs of *Npr1*$^{+/+}$ mice or *Npr1*$^{-/-}$ mice. Each bar gives the mean ± S.E.M. The number in parentheses denotes the number of independent pairs of animals tested. *, $P < 0.05$ relative to wild-type; **, $P < 0.01$; ***, $P < 0.001$.

though the results do not allow us to definitively rule out a multiple-site model. Nevertheless, these results again argue strongly that the BNP responses are not mediated by NPRB. First, the EC_{50} for BNP activation of recombinant rodent NPRB [26,27,38] is nearly two orders of magnitude higher than the EC_{50} for the BNP responses that we observe in *Npr1*$^{-/-}$ testis. Second, recombinant NPRB is approximately equally responsive to ANP and BNP [26,27,38], whereas the receptor that mediates the high affinity BNP responses in *Npr1*$^{-/-}$ testis is clearly quite insensitive to ANP.

Taken together, the results in Figures 4–8 support two principal conclusions: (1) NPRA is the most abundant rGC capable of generating cGMP-dependent responses to BNP, and (2) certain tissues display an additional, less prominent BNP-selective activity that is mediated by a high affinity mechanism distinct from both NPRA and NPRB.

The basal rate of cGMP synthesis reflects the complement of membrane GCs expressed by each tissue

A low rate of activity in the absence of ligand is characteristic of many receptors, including rGCs. As can be shown in Figures 4–6, the basal rate of cyclase activity observed in tissues derived from *Npr1*$^{-/-}$ animals is often much lower than that observed in corresponding tissues from *Npr1*$^{+/+}$ animals (note the differences in scale). We have pooled together all of the measurements that were made in the course of our studies, and have used these results to determine the basal cyclase activity for each *Npr1*$^{+/+}$ and *Npr1*$^{-/-}$ tissue (Figure 9). In some tissues (for example, lung, forebrain and liver) the basal rate at which cGMP is synthesized by *Npr1*$^{-/-}$ and *Npr1*$^{+/+}$ membranes is not statistically different, implying only a minor contribution to basal activity by NPRA. In contrast, adrenal gland, kidney and testis show a large proportional decrease of basal activity in mutant membranes relative to wild-type membranes, consistent with a dominant role for NPRA in controlling cGMP metabolism in these tissues. Furthermore, the amount of basal cyclase activity remaining in each *Npr1*$^{-/-}$tissue reveals the extent to which membrane cyclases other than NPRA are active in that tissue. The rank order for residual basal cyclase activity (pmol of cGMP synthesized/min per mg of protein) in tissues from *Npr1*$^{-/-}$ animals is as follows: lung, 6.1 ± 1.3 ($n = 25$); colon, 4.0 ± 0.6 ($n = 18$); adrenal gland, 2.4 ± 0.6 ($n = 9$); forebrain, 1.5 ± 0.3 ($n = 8$); kidney, 1.0 ± 0.1 ($n = 23$); testis, 0.9 ± 0.1 ($n = 12$); heart, 0.6 ± 0.2 ($n = 8$); liver, 0.5 ± 0.1 ($n = 11$); aorta, 0 ($n = 2$).

At least part of the basal activity remaining in many tissues must reflect the contribution of NPRB. Based on responsiveness to CNP (Figure 7), NPRB is expressed prominently in adrenal gland, lung, kidney, testis, liver, colon and heart. In contrast, CNP has relatively little effect on membranes isolated from forebrain. Interestingly, in wild-type forebrain membranes there is also only a small increase in cGMP after stimulation with ANP or BNP (Figures 4 and 5), and 97% of the relatively high basal GC activity remains after NPRA deletion (Figure 9). This suggests that membrane-associated GCs other than NPRA and NPRB are prevalent in this tissue. Since neither the residual response to BNP nor any of the other currently identified members of the rGC family are prominently expressed in forebrain, it seems likely that novel rGCs (or perhaps other, as yet uncharacterized, GC isoforms that are not in the rGC family) remain to be discovered in the nervous system.

DISCUSSION

Although it is well accepted that ANP and BNP, acting through NPRA, play important roles in blood pressure and fluid volume homoeostasis [1–9,31], significant gaps remain in our understanding of this critical endocrine pathway. For example, we lack a systematic 'functional' evaluation of NPRA activity across a representative spectrum of tissues. Such measurements are of significant interest, given the known dependency of NPRA activity on phosphorylation state [36]. A related issue, important for understanding how NP signalling pathways influence blood pressure in the intact animal, is whether the activity of NPRB, a structurally similar rGC, can be up-regulated (through phosphorylation or some other mechanism) to compensate for deficiencies in NPRA signalling. An additional long-standing, unanswered question is whether BNP (which activates NPRA with significantly lower potency than does ANP) might actually serve as the preferred agonist at some alternative, yet-to-be-identified receptor. Gene targeting has been used to disrupt the murine gene encoding NPRA [7,31], providing an optimal experimental system in which to address these issues.

The most novel finding of the present study is the observation that certain tissues from NPRA-deficient animals (notably testis and adrenal gland) retain a significant level of responsiveness to BNP, though they have lost the ability to respond to physiologically relevant levels of ANP. This strongly implies the existence of a BNP-preferring receptor that is preferentially expressed in steroid-producing tissues. The cGMP-producing capacity of this BNP-preferring pathway is low (approx. 10% of the capacity of the NPRA pathway), suggesting either that we have not yet identified the ligand that activates it most effectively, or that its signalling function may be carried out only within a subset of cells in each responsive tissue.

A number of observations suggest that these residual responses are not mediated by any of the currently-known rGCs. (1) They cannot be mediated by NPRA, since the NPRA gene has been ablated in the mutant animals, and we find no evidence that this

mutation is 'leaky'. (2) Although NPRB responds weakly to both ANP and BNP, its EC_{50} for activation by BNP is greater than 1 μM [26,27,38], at least two orders of magnitude higher than the EC_{50} of the residual BNP responses mediated by the putative BNP receptor. Furthermore, we find an obvious mismatch between the ability of mutant tissues to respond to CNP (the most potent known ligand for NPRB) and BNP, implying that the two peptides are acting through distinct receptors. (3) None of the other known mammalian rGCs are significantly sensitive to ANP or BNP, although one of them (GC-G) does respond very weakly to ANP [39]. GC-G is, however, completely insensitive to BNP, and its tissue distribution in rats (high in lung, and undetectable in testis and adrenal gland) is inconsistent with the tissue distribution of the residual BNP responses that we observe in *Npr1*$^{-/-}$ mice.

We know little about the receptor that mediates the residual BNP responses, other than that it can enhance net cGMP synthesis, and that it prefers BNP over ANP. If this receptor is a member of the rGC family, its low abundance may provide an explanation for why it has not been cloned from any of the cDNA libraries used in previous studies. Alternatively, it could represent a novel type of membrane GC that has not been identified in standard library screens because it lacks sufficient structural homology to the rGC family. As an additional possibility, it may be that the properties of a known rGC, such as NPRB, have been altered in specific tissues (for example, by post-translational modification or by association with an accessory subunit), so that the modified receptor has become capable of mediating the responses that we observe. Finally, the receptor could be coupled in an inhibitory way to a phosphodiesterase that breaks down cGMP. If so, the targeted phosphodiesterase must be membrane-associated and resistant to IBMX, a broad-spectrum phosphodiesterase inhibitor that is routinely included in our incubation mixture.

If a BNP-preferring signalling pathway does indeed exist, then it is likely to be affiliated with physiological responses distinct from those that are regulated by NPRA. In this regard, it is worth noting that the phenotypes of ANP and BNP knockout animals are not equivalent. ANP knockout animals develop hypertension and cardiac hypertrophy, without reported signs of cardiac fibrosis [40], whereas BNP knockout animals frequently develop fibrotic lesions throughout their ventricles, but have normal blood pressures, normal sized hearts and normal circulating levels of ANP [41]. This strongly suggests that there are aspects of BNP signalling that cannot be substituted by ANP acting through NPRA, and provides indirect support for the existence of a BNP-preferring receptor. If the role of this receptor is to suppress cardiac fibrosis (as implied by the phenotype of the BNP deficient animals), then the BNP-selective responses that we have observed in steroid-producing tissues are of particular interest, given the well known relationship between steroids and fibrosis [42]. Together, these observations suggest that one unique function of BNP may be to limit fibrosis by controlling steroid production.

A second goal of our studies was to determine if rGC activity is maintained in proportion to protein levels, or whether feedback mechanisms might alter enzyme activity to keep it at, or near, a biological optimum in specific tissues, or in response to changes in blood pressure. Our functional studies show that the tissue distribution of NPRA-mediated cGMP synthesis agrees well overall with the distribution of immunoreactive NPRA. By both measures, expression is high in adrenal gland, kidney and lung, and lower, but still abundant in testis and liver. This expression pattern is substantiated by a spectrum of well established physiological actions of ANP in kidney [6,31,43], adrenal gland [44], lung [45], testis [46] and liver [47–49]. The correlation that we observe between NPRA protein levels and NPRA-mediated enzyme activity indicates that, under normal circumstances, regulatory mechanisms (such as protein phosphorylation) do not significantly adjust the function of NPRA in specific tissues. Somewhat surprisingly, this also appears to be the case under hypo- or hypertensive conditions. In a previous study [9], we used mice with 1, 2, 3 and 4 copies of the gene encoding NPRA to evaluate the relationship between gene-copy number, blood pressure and NPRA-dependent enzyme activity. Despite the fact that the genetically modified animals show significant differences in blood pressure, they maintain a strikingly linear relationship between NPRA gene-copy number and ANP-stimulated cGMP synthesis. As an alternative, a mouse with altered NPRA expression could also normalize its blood pressure through compensatory changes in NPRB activity. However, our current measurements indicate that there is no difference in NPRB rGC activity in the lungs of *Npr1*$^{-/-}$ mice relative to *Npr1*$^{+/+}$ mice, despite the fact that this tissue is highly vascularized. Taken together, these results suggest that neither protein expression levels nor enzyme activity of peptide-sensitive rGCs is altered to compensate for chronic deviations in blood pressure.

In summary, our studies provide evidence that BNP can alter cGMP levels in mouse tissues by at least two different high affinity mechanisms. One mechanism employs NPRA, a conventional high-capacity rGC that is particularly abundant in adrenal gland, lung, kidney, testis and liver. The second mechanism activates an as yet unspecified lower-capacity response that can be observed only in adrenal gland and testis. Although the physiological significance and biochemical components of this second mechanism remain to be established, its existence does reinforce the notion that, consistent with their independent structures, ANP and BNP are likely to carry out at least some independent actions. In addition, our results indicate that, unlike many other receptors that play a role in blood pressure homoeostasis, the activity and expression levels of the rGCs are not tightly regulated by feedback mechanisms. This leads to the prediction that any mutation leading to loss or gain of NPRA or NPRB function should alter the cellular response to the NPs in direct proportion to the activity of the mutant gene product, reinforcing the notion that polymorphisms in the genes encoding the rGCs may contribute to the genetic basis of hypertension [9].

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