Neurokinin 1 receptors and neprilysin modulation of mouse bladder gene regulation

IGOR DOZMOROV, MARCIA R. SABAN, NORMA P. GERARD, NGOC-BICH NGUYEN, MICHAEL CENTOLA, AND RICARDO SABAN


Dozmorov, Igor, Marcia R. Saban, Norma P. Gerard, Bao Lu, Ngoc-Bich Nguyen, Michael Centola, and Ricardo Saban. Neurokinin 1 receptors and neprilysin modulation of mouse bladder gene regulation. Physiol Genomics 12: 239–250, 2003. First published December 19, 2002; accepted in final form 18 December 2002.—Neurokinin 1 (NK1) receptors play a fundamental role in neurogenic inflammation. We sought to determine the mechanisms downstream from NK1 receptor (NK1R) activation using cDNA arrays and a novel statistical method to analyze gene expression. We used female NK1 receptor (NK1R) and wild-type (WT) mice that were sensitized actively by intraperitoneal injections of dinitrophenol 4 (DNP4)-human serum albumin. Cystitis was induced by intravesical instillation of antigen of DNP4-ovalbumin, and control mice were challenged with saline. At 1, 4, and 24 h after instillation, bladders were removed for RNA extraction (n = 3), and 2 replicate of RNA extraction (n = 3), and 3 morphological analysis (n = 6). For cDNA array experiments, three bladders from each group were homogenized, and total RNA was obtained. DNase-treated RNA was reverse-transcribed to cDNA, labeled with [α-32P]dATP and hybridized to Atlas Mouse 1.2 Arrays (Clontech). After calculating the mean and SD for background spots, each experimental value was assigned a normalized score \( S \) using the formula \( S' = (S - Av)/SD \), where \( S' \) is the original pixel value, and \( Av \) and SD are the mean and standard deviation of background spots, respectively. Only genes that expressed 3 SD values above background were considered. Hypervariable genes were sorted by cluster analysis. Matrices of correlation coefficients were calculated and represented in a connectivity mosaic. As results, we found that in WT mice the most prominent gene cluster had neprilysin in a central position and positively correlated to a group of activator protein-1 (AP-1)-responsive genes, including laminin-α3, tissue plasminogen activator 11, fos-B, and TNF-β. In WT mice, antigen-induced bladder inflammation led to a downregulation in neprilysin expression. In contrast, NK1R \( \sim R \) mice failed to mount an inflammatory reaction and presented neprilysin negatively correlated with the same genes described in WT. In conclusion, this work indicates an overriding participation of NK1R and neprilysin in bladder inflammation, provides a working model for the involvement of AP-1 transcription factor, and evokes testable hypotheses regarding the role of NK1R and neprilysin in inflammation.

INFLAMMATION UNDERLINES all major bladder pathologies and represents a defense reaction to injury caused by physical damage, chemical substances, microorganisms, or other agents (38). During inflammation, a cascade starts on urothelial cells which relay the information to sensory nerves (3). Within the bladder wall, sensory nerves release substance P (SP) (5, 40) and its actions are controlled by neprilysin [neutral endopeptidase (NEP), EC 3.4.24.11, or CD10] expressed on the cell surface of the urothelium, submucosa, and detrusor muscle (40, 51).

SP activation of G-protein-coupled neurokinin (NK) receptors (49) induces a sequential activation of signaling pathways leading to the production of both pro- and anti-inflammatory mediators (38). NK1 receptors (NK1R) are the predominant subtype involved in inflammation in general (49) and also in bladder plasma extravasation. In this context, an upregulation of NK1Rs was found in bladder inflammation (45), and bladder biopsies from cystitis patients present an increase in NK1R density (26). Others have shown that an increase in NK1R numbers may underlie persistent pain, such as that observed during chronic bladder inflammation (1). The use of NK1R \( \sim R \) mice confirmed a central role for SP in several models of inflammation including cystitis (42). In addition, the NK1R \( \sim R \) mouse was used to reveal other inflammatory pathways such as the involvement of calcitonin gene-related peptide (CGRP) (14) in mediating neurogenic plasma extravasation. However, the mechanisms downstream of NK1R activation remain undefined. Therefore, the aim of this study was to characterize the cascade of events downstream of NK1Rs linking the stimulus to inflammation. For this purpose we combined an elegant animal model with the power of gene expression profiling.

Gene expression profiling is an effective means of comprehensively cataloging the molecular conse-
quences of specific cellular responses. However, data analysis is complicated simply by the size of the data sets. Commonly used statistical thresholds for identification of differentially expressed genes result in hundreds of false-positive identifications in an expression profiling experiment in which thousands of independent comparisons are performed. Although the use of stringent thresholds corrects for this error, these result in false-negative selections and a tradeoff in sensitivity vs. specificity. The method introduced here, denoted “associative analysis,” supplements the standard procedure of multiple paired comparisons by associating the expression level of each gene in the experimental group with a family of similarly and stably expressed genes in the control group. This associative membership analysis enhances the sensitivity of analysis greater than previously developed modifications of the t-test described in literature (31) and increases the number of differentially expressed genes identified without significantly increasing the misidentification of false positives (10). Herein, we describe the first use of gene expression profiling using the associative method combined with a novel clustering procedure based on the calculation of positive and negative correlations among groups of hypervariable genes. This clustering method prioritizes cluster definitions using a simple yet powerful parameter-cluster size.

Our analysis revealed a dramatic change in organ-specific inflammatory signaling events of wild-type (WT) and NK1-R knockout mice (NK1R−/−). Our findings provide evidence of a strong alteration in the inflammatory pathway in the NK1R−/− mice, where several activator protein-1 (AP-1)-responsive genes were released from regulatory control of NEP.

METHODS

Animals

All animal experimentation described here was performed in conformity with the “Guiding Principles for Research Involving Animals and Human Beings” (OHSC Animal Care and Use Committee protocol no. 00-109). Groups of 10- to 12-wk-old female mice were used in these experiments. NK1R−/− and WT littermate control mice were maintained by Dr. Norma P. Gerard. The colonies at OHSC were genotyped as described previously (6).

Antigen Sensitization Protocol

All mice in this study were sensitized with 1 μg DNP3-human serum albumin (HSA) in 1 mg alum on days 0, 7, 14, and 21, intraperitoneally. In normal mice, this protocol induces sustained levels of IgE antibodies up to 56 days post-sensitization (42). One week after the last sensitization, cystitis was induced.

Induction of cystitis

Cystitis was induced as we described previously (38, 42). Briefly, sensitized WT and NK1R−/− mice were anesthetized (ketamine 40 mg/kg and xylazine 2.5 mg/kg ip), then transurethrally catheterized (24 gauge, 3/4 in., Angiocath; Becton-Dickinson, Sandy, UT), and the urine was drained by applying slight digital pressure to the lower abdomen. The urinary bladders were instilled with 150 μl of one of the following substances: pyrogen-free saline or dinitrophenol 4 (DNP4-) ovalbumin (1 μg/ml). All substances were infused at a slow rate to avoid trauma and vesicoureteral reflux (38). To ensure consistent contact of substances with the bladder, infusion was repeated twice within a 30-min interval, and a 1-ml tuberculin (Tb) syringe was maintained into the catheter for at least 1 h. After Tb, the catheter was removed, and mice were allowed to void normally. At 1, 4, and 24 h after instillation, mice were killed with pentobarbital (100 mg/kg ip), and bladders were removed rapidly. Bladders were randomly distributed into the following groups: 1) RNA extraction (n = 3), 2) replicate of RNA extraction (n = 3), and 3) and morphological analysis (n = 6). Therefore, two pools of RNA were generated that permitted two different cDNA hybridizations per experimental group.

Altersations at Histological Level

The urinary bladder was evaluated for inflammatory cell infiltrates, mast cell numbers, and the presence of interstitial edema. A semi-quantitative score using defined criteria of inflammation severity was evaluated to quantify cystitis (38, 42). A cross section of bladder wall was fixed in formalin, dehydrated in graded alcohol and xylene, embedded in paraffin, and cut serially into four 5-μm sections (8 μm apart) to be stained with hematoxylin and eosin (H&E) and Giemsa. Histology slides were scanned using a digital camera (model DXM1200, Nikon) mounted on a microscope (model Eclipse E600, Nikon). Image analysis was performed using a Meta-Morph Imaging System (Universal Imaging, West Chester, PA). The severity of lesions in the urinary bladder was graded as follows: 1+, mild (infiltration of a low number of neutrophils in the lamina propria, and little or no interstitial edema); 2+, moderate (infiltration of moderate numbers of neutrophils in the lamina propria, and moderate interstitial edema); 3+, severe (diffuse infiltration of moderate to large numbers of neutrophils in the lamina propria and severe interstitial edema) (38, 42).

Minimum Information About Microarray Experiments (MIAME)

Objective. Our objective in this study was to determine the time course of gene expression in control and antigen-inflamed WT and NK1R−/− mice.

Array design. Mouse 1.2 Arrays (catalog no. 7853-1; Clontech, Palo Alto, CA) containing 1,177 known mouse genes, 3 negative controls, and 9 housekeeping genes. For a complete list of genes present in this array see http://www.clontech.com/atlas/genelists/index.html.

Animal numbers. Female WT and NK1R−/− mice C57BL/6J mice were instilled with antigen (in sensitized mice), or saline. At 1, 4, and 24 h following stimulation, the urinary bladders were randomly distributed into the following groups: 1) RNA extraction (n = 3), 2) replicate of RNA extraction (n = 3), and 3) and morphological analysis (n = 6).

Sample preparation for cDNA expression arrays. We used the same technology as we described before (38, 37). Briefly, three bladders from each group were homogenized together in Ultraspec RNA solution (Biotecx Laboratories, Houston, TX) for isolation and purification of total RNA. Mouse bladders were pooled to ensure adequate RNA for array analysis. The justification for this approach is that there is not sufficient RNA in a single mouse bladder for performing cDNA array experiments, and the step of purification reduces the amount of total RNA. RNA was DNase-treated according to manufacturer’s instructions (Clontech Laboratories), and
the quality of 10 μg was evaluated by denaturing formaldehyde/agarose gel electrophoresis.

Mouse cDNA expression arrays. cDNA probes prepared from DNase-treated RNAs obtained from each of the experimental groups were hybridized simultaneously to Atlas Mouse 1.2 Arrays (catalog no. 7853-1, Clontech; for a complete list of genes present in this array see http://www.clontech.com/atlas/genelists/index.html). Briefly, 5 μg of DNase-treated RNA was reverse-transcribed to cDNA and labeled with [α-32P]dATP, according to the manufacturer’s protocol (Clontech). The radioactively labeled complex cDNA probes were hybridized overnight to Atlas Mouse 1.2 Arrays (Clontech) using ExpressHyb hybridization solution with continuous agitation at 68°C. After two high-stringency washes, the hybridized membranes were exposed (at room temperature) to a ST Cyclone phosphor screen overnight.

Signal quantification. The phosphor imaging screen contains phosphor crystals that absorb the energy emitted by the radioactivity of the sample and re-emit that energy as a blue light when excited by a red laser. Results are presented as digital light units (DLU). Spots on the arrays were quantified by BD AtlasImage 2.7 software (Clontech). The results were placed in an Excel spreadsheet.

Data Normalization and Analysis

Data among experiments was first normalized to background. Histograms of all intensity values demonstrated the presence of the normally distributed spots corresponding to cDNA targets that do not hybridize to a detectable extent with the labeled test probes (Fig. 1A). These signals were due to noise and therefore fit a random distribution whose prop-

![Fig. 1. Normalization of the gene expression profile to its own background. A: histograms showing the expression levels of the 1,176 cDNA targets derived from two samples (S1 and S2) of wild-type (WT) mice treated with saline (control). B: log transformation of the data presented in A. Normal distributions of background spots had a mean = 0 and SD = 1. Expressed genes were defined as those whose expression levels were 3 SD values above the mean background level and were used for the final adjustment of profiles by robust regression analysis (C). C: adjustment of the normalized profiles to each other by robust regression analysis of genes expressed above background. The parameters of the regression line excluded spots whose residuals lay beyond normally distributed residuals of expressed genes. Data of sample S2 were transformed to promote the final position of the regression line passing through the origin and presenting a slope = 1. D: data of the plot C presented in transformed coordinates, where abscissa is gene expression and ordinate is the deviation of expression from the equity position (residual). On right, a graphic represents the normal distribution for the majority of deviations. Genes whose deviations distributed beyond of the indicated statistical threshold were treated as differentially expressed (open circles).
Properties (mean and SD) were proportional to the total amount of signal on the array and hence can be used for data normalization among array experiments. The mean and variance of the intensity levels of nonexpressed genes was estimated by excluding expressed genes from the distribution using a nonlinear curve-fitting algorithm in which a given gene expression value exceeding the mean of the distribution of nonexpressed genes (±2 SD) was discarded and a new mean and SD determined for the remaining set. This process was repeated in an iterative manner until no additional spots were excluded, and the resulting nondiscarded points (typically between 500 and 600 of the initial set of 1,176) represented the set of nonexpressed genes (Fig. 1B).

After calculating the mean and SD for the set of these normally distributed background spots, each experimental value was assigned a normalized score $S$ using the formula $S' = (S - \text{Av})/\text{SD}$, where $S$ is the original pixel value for the spot, and Av and SD are the mean and standard deviation, respectively, of the set of background spots. (After normalization, the $S'$ distributions of all experiments had a mean of zero and SD = 1 (Fig. 1B)). Only genes expressed above background were used for the second normalization step. Expressed genes were defined as those whose expression levels were 3 SD values above the mean background level as described (35).

Data was further normalized by linear regression analysis using only genes expressed above background. Expressed gene values were corrected such that the slope of the line was equal to 45° and passed through the origin (Fig. 1C). Values were log-transformed to avoid the influence of the small number of very intense spots in the subsequent steps of analysis. It was therefore necessary to assign a minimum value to any gene with a normalized expression level equal to or below zero.

Identification of Differentially Expressed Genes

Most gene expression levels did not change among these groups. Therefore, after exclusion of background noise and log transformation, it was observed that the majority of genes had expression residuals that fit a normal distribution, as revealed by Kolmogorov-Smirnov test (10). Outliers from this group were identified and excluded using the same iterative procedure described above such that the set of similarly expressed genes can be utilized for identification of differentially expressed genes (Fig. 1D).

For each expressed gene, residuals were determined as the differences between gene expression level and the average of expression values obtained in the control group. This statistical measurement of gene expression variability was then used for differential gene expression analysis. A plot of the residuals of the WT mouse, the reference group, and the knockout mouse reveals a differential gene expression (Fig. 2, A–C). Genes that were differentially expressed in the experimental group appear

![Fig. 2. Deviations of gene expression.](https://www.physiolgenomics.org/)

Gene expression was rescaled to the averaged data obtained in WT mice treated with saline (control group). A: control group residuals; variability of genes within the control group. B: reference group; the same data presented in A, after exclusion of hypervariable genes (procedure based on F criterion as described in METHODS). C: experimental group residuals; deviation from normal control averages of gene expressions in NK1R−/− mice samples.
to have highly variant distributions of expression represented by significantly variant residuals (Fig. 2C). These differentially expressed genes were identified first by a paired t-test and the commonly accepted significance threshold of \( P < 0.05 \). Taken in consideration that a significant proportion of the genes were falsely determined, we used a second method of selection. For this purpose, we applied an “associative t-test” in which the replicated residuals for each gene of the experimental group were compared with the entire set of residuals from the reference group defined above. In this analysis, the null hypothesis was checked to determine whether gene expression in the experimental group was associated with the reference group. The significance threshold was corrected in the associative t-test to make improbable the appearance of false-positive determinations. Since hundreds of comparisons are made, this does not result in loss of sensitivity. Comparison of the selections from the paired t-test and associative t-tests were then used to classify the differentially expressed genes as 1) likely false positives (these are genes selected as differentially expressed by the paired t-test with \( P < 0.05 \), but not by the associative t-test), 2) real positives (selected in both tests), and 3) potential positives (genes selected in the associative test only).

**Comparative Cluster Analysis of Gene Expression Dynamics**

Comparative pair-wise correlations were calculated among gene expression levels such that groups of genes that exhibited temporally correlated behavior could be identified in the WT and knockout groups. The clustering procedure was based on the Pearson correlation and consists of the following steps: 1) gene expression normalization, log transformation, and rescaling as described above; 2) identification of, and limiting subsequent analyses to, genes expressed above background (3 SD values above background noise) in at least one time point; 3) identification of, and limiting of subsequent analysis to, genes with expression levels that vary among time points (based on an F criterion). These genes are denoted as “hypervariable.”

**Determination of Connectivity for Each of These Hypervariable Genes**

Connectivity was defined as the number of genes whose expression behavior among the time course was correlated with a given gene. The appropriate threshold for the correlation coefficients used to define connectivity was calculated using a simulation study. Hypervariable genes of the control group were sorted by their connectivity, and the clustering process was started with genes of higher connectivity. The gene of higher connectivity and all genes correlated with it comprise cluster 1. The next gene of higher connectivity not belonging to the first cluster and the genes correlating with it comprise cluster 2, and the process continued until all genes were analyzed. This method was developed to maximize the size of the clusters such that the most comprehensive global group dynamics were revealed. Genes that appeared in more than one cluster were considered likely functional links among these clusters. Genes that had zero connectivity did not belong to any cluster. Hypervariable genes expressed in tissues isolated from WT and knockout mice were clustered as described above. Matrices of correlation coefficients were calculated for these clusters and represented in a graphical output termed “connectivity mosaic.” This mosaic permits a visual inspection of correlated and noncorrelated genes (Fig. 3A). To establish a robust statistical criterion for clustering, the effect of random chance for the correlation appearance was empirically determined using a Monte Carlo simulation experiment (10). The set of expressions was simulated with random numbers having exactly the same averages and standard deviations as genes of the WT and knockout groups. Connectivity of these simulated expressions was calculated with different correlation coefficient thresholds (CC). Thresholds were chosen that excluded the occurrence of false positives. If a correlation was observed in only one group, then a threshold (CC = 0.995) was required to maintain statistical significance. However, for correlations identified in both the groups, this reproducibility of the correlation permitted the use of a lower threshold (CC = 0.9). These estimations were averaged from three individual simulation experiments. As-
associations weaker than threshold strength were removed, leaving a network of highly correlated genes.

RESULTS

Morphology

We first examined whether the bladder of sensitized mice developed inflammation secondary to antigen challenge. Confirming our previous observations (42), bladders isolated from sensitized WT mice that were challenged with saline did not present any sign of edema or neutrophil infiltration. Sensitized WT mice challenged with antigen developed an inflammatory response characterized by vasodilation, edema, intense polymorphonuclear neutrophil (PMN) infiltration in the mucosa and submucosal layers (Fig. 3, A and B), and activation of resident mast cells (42). This inflammatory response, observed 24 h after antigen stimulation, had predominant characteristics of acute inflammation based on the strong vascular component, predominance of PMNs, and near absence of macrophages/monocytes. In contrast, histological quantification of the bladder sections indicated that NK1R−/− mice had significant attenuation of congestion and edema of the mucosa (Fig. 3A) as well as reduced PMN infiltration in response to antigen challenge (Fig. 3B). Histologically, antigen-challenged bladders of these mice appeared to be no different from sensitized WT exposed to saline (42).

Reproducibility of Array Hybridization

We previously presented evidence of the reproducibility of gene-array methodology for the analysis of bladder inflammatory genes (37, 38) and verified the results using RNase protection assay (37). In the present work, we determined the reproducibility of our hybridization technique by performing regression analysis from values obtained with two different pools of RNA. The calculated correlation coefficients for raw data of WT and NK1R mice challenged with saline were 0.9838 and 0.9718, respectively.

Differential Gene Expression Between WT and NK1R−/− Mice

We next investigated whether the null mutation would reveal genes that were under the negative control of NK1R−/− gene. Table 1 indicates genes that were found differentially expressed in bladders isolated from WT and NK1R−/− regardless of the treatment (saline or antigen) or time (1, 4, and 24 h) following intravesical challenge. Table 1A lists genes that were expressed only in bladders removed from NK1R−/−, and Table 1B lists genes overexpressed in NK1R−/−. Those genes are strong candidates to be under the negative influence of NK1R. In contrast, Table 1C lists genes that were expressed only in WT mice and Table 1D lists genes overexpressed in WT mice. Those genes are strong candidates to be dependent on a positive influence of NK1R activation.

Network of Highly Correlated Genes

We next identified groups of genes whose expression levels varied in a temporally coordinated manner. For this purpose, we developed a novel statistically robust clustering method that identifies both positively and negatively correlated behavior. In this method, the

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Table 1. Genes differentially expressed in WT and NK1R−/− mice

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Gene Name</th>
<th>Gene Group</th>
<th>GenBank Acc. No.</th>
</tr>
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<tbody>
<tr>
<td>A: Genes expressed only in NK1R−/− mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRCI</td>
<td>macrophage mannose receptor</td>
<td>Receptors</td>
<td>Q61830</td>
</tr>
<tr>
<td>CPR</td>
<td>NADPH-cytochrome P-450 reductase</td>
<td>Apoptosis-associated proteins</td>
<td>P37040</td>
</tr>
<tr>
<td>IKK-γ</td>
<td>IkB kinase gamma subunit</td>
<td>Intracellular kinase network members</td>
<td>O88522</td>
</tr>
<tr>
<td>DSG3</td>
<td>desmoglein 3</td>
<td>Cell adhesion receptors and proteins</td>
<td>O35902</td>
</tr>
<tr>
<td>B: Genes overexpressed in NK1R−/− mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAMA2</td>
<td>laminin alpha 2 subunit precursor</td>
<td>Cytoskeleton and motility proteins</td>
<td>Q60675</td>
</tr>
<tr>
<td>CD31</td>
<td>platelet endothelial cell adhesion molecule 1</td>
<td>Cell-cell adhesion receptors</td>
<td>Q08481</td>
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<tr>
<td>Bm-3.2</td>
<td>Pou/ transcription factor</td>
<td>Transcription activators and repressors</td>
<td>Q63934</td>
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<td>HSP86</td>
<td>heat shock 86-kDa protein</td>
<td>Heat shock proteins</td>
<td>P07901</td>
</tr>
<tr>
<td>LAMB1-1</td>
<td>laminin beta 1 subunit 1 precursor</td>
<td>Cytoskeleton and motility proteins</td>
<td>P02469</td>
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<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion protein 1</td>
<td>Matrix adhesion receptors</td>
<td>P29533</td>
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<tr>
<td>GBP1</td>
<td>guanine nucleotide binding protein</td>
<td>G proteins</td>
<td>P04894</td>
</tr>
<tr>
<td>cathepsin H</td>
<td>cathepsin H</td>
<td>Cysteine proteases</td>
<td>P49935</td>
</tr>
<tr>
<td>NEFL</td>
<td>neurofilament triplet L protein</td>
<td>Cytoskeleton and motility proteins</td>
<td>P08551</td>
</tr>
<tr>
<td>C: Genes expressed only in WT mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGH2 synthase</td>
<td>prostaglandin I2 (prostacyclin) synthase</td>
<td>Prostaglandins</td>
<td>O35074</td>
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<tr>
<td>IGFBP4</td>
<td>insulin-like growth factor binding protein 4</td>
<td>Growth factors, cytokines, and chemokines</td>
<td>P47879</td>
</tr>
<tr>
<td>FLIP-L</td>
<td>FLICE-like inhibitory protein long form</td>
<td>Apoptosis-associated proteins</td>
<td>O35507</td>
</tr>
<tr>
<td>PGP4</td>
<td>fibroblast growth factor 4 precursor</td>
<td>Growth Factors, cytokines, and chemokines</td>
<td>P11403</td>
</tr>
<tr>
<td>BCLW</td>
<td>B-cell lymphoma protein W</td>
<td>Bcl family proteins</td>
<td>P70345</td>
</tr>
<tr>
<td>D: Genes overexpressed in WT mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GABT3</td>
<td>GABA transporter 3</td>
<td>Symporters and antiporters</td>
<td>P31650</td>
</tr>
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</table>

WT, wild type. NK1R, neurokinin 1 receptor.
Statistical criteria were established at two stages: the first identified genes that exhibit hypervariable behavior over time, and the second identified groups of genes with similar patterns of expression. These strict statistical criteria eliminated the misidentification of group members due to chance. In addition, the size of the clusters, denoted connectivity, was used to prioritize a given gene assignment within groups such that the most prominent aspects of coregulation was identified. Matrices of correlation coefficients were calculated and represented in a graphical output termed connectivity mosaic. The latter permitted a visual identification of the coordinated genes behavior in the WT and NK₁R⁻/⁻ mice (Fig. 4, A and B; Table 1). The mosaics indicate that NEP switches from strongly positive correlation to negative correlation between WT (Fig. 4A) and NK₁R⁻/⁻ (Fig. 4B). Another gene that switched to negative correlation was endoglin (CD105). Finally, the majority of genes that exhibited temporally correlation did not present a differential expression between WT and NK₁R⁻/⁻ mice.

Network Construction

Based on these results, we built a network of highly correlated genes that is represented by two clusters (Fig. 5 and the table in Fig. 6). In the primary cluster, NEP/CD10 (M81591) occupies a central position correlating positively with Lfc, LAMA3, and tissue plasminogen activator 11 (TPA-11). Interestingly, NEP correlations switched from positive in WT (Fig. 5A) to a negative in NK₁R⁻/⁻ (Fig. 5B). These data were confirmed in an independent analysis of differential gene expression demonstrating that antigen-induced in-

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**Fig. 4.** Mosaic of correlation coefficients for the hypervariable genes in WT (A) and NK₁R⁻/⁻ mice (B). White lines in A indicate the borders of three clusters of tightly interconnected genes. Colored lines and spots beyond the clusters represent positively linked genes (red) belonging to two or more clusters (gene 5 for example), or negative linked genes (blue). Genes that had a positive correlation through time were represented in graded shades of red and genes negatively correlated in graded shades of blue, whereas absence of correlation is indicated in green.
flammmation induced a downregulation in NEP expression only in WT mice (Fig. 6). Another interesting correlation was found between CGRP receptor, Maf 1, and TPA-11. In the same cluster there was a positive correlation between the transcription factor ElfI and TNF-β.

The secondary cluster contains collagen 9α2 (COL9A2), von Hippel-Lindau syndrome homolog (VHLH), mouse adenylate cyclase inhibitory protein (MACIP), cytochrome P-450 oxioreductase (CPR), and protein tyrosine phosphatase (PTP). The relationship between genes of the secondary cluster and the connection between the two clusters by the pairs NEP and COL9A2 and TGF-β and VHLH has been less documented.

DISCUSSION

The NK1R−/− provided further evidence that SP plays a mandatory role in bladder inflammation. Before searching for pathways downstream of NK1R activation, we analyzed whether NK1R−/− mice present a differential gene expression. Our results indicate that NK1R−/− failed to express certain genes normally found in the WT mice, including PGI2 (prostaglandin I2 synthase, AB001607); IGFBP4 (insulin-like growth factor binding protein 4 precursor, X81582); FLIP-L (FLICE-like inhibitory protein long form, U97076); FGF-K (mouse K-fibroblast growth factor or FGF4, M30642); BCLW (B-cell lymphoma protein W, U59746). In addition, the GABA transporter 3 was overexpressed in WT mice relative to knockout mice. An interesting feature of prostaglandins is the sensitization of neurons to pain (45), and PGI2 along with PGE2 induces the release of SP from the dorsal root ganglia (45). However, the role of prostaglandins in neurogenic inflammation is not entirely clear, and it remains to be determined whether a decrease in PG production is responsible for the overall absence of
inflammation in NK1R−/− mice. IGFBPs seem to participate in bladder responses to diabetes (7). Little is known about the interaction of SP and IGFBP4. Among the tachykinin receptors, only the NK1R system was involved in the synergistic effect of SP and IGF-I on corneal epithelial migration (32). Together these results indicate that interactions between SP and IGFBP4 may be tissue specific and indicate the need for investigation of such association within the urinary bladder physiopathology.

FLIP-L is an inhibitor of apoptosis which is predominantly expressed in muscle and lymphoid tissues. FLIP-L is expressed during the early stage of T cell activation, but disappears when T cells become susceptible to Fas ligand-mediated apoptosis. Our findings strongly correlated the absence of both FLIP and cystitis in the NK1R−/− and therefore emphasize the need for determination of FLIP expression in cystitis.

Interestingly, a group of genes was expressed only in NK1R−/− mice and may represent a repressor function of NK1R on gene regulation. Those included MRC1 (macrophage mannose receptor, Z11974), CPR (NADPH-cytochrome P-450 reductase, D17571), NEMO (IKKβ kinase subunit, AF069542), and DSG3 (desmoglein 3, U86016). NEMO is a regulatory protein of the IKKα and IKKβ kinases. Both kinases modulate IκB activity and consequently NF-κB translocation and expression of genes involved in inflammation. Because of its regulatory properties, NEMO is the target of newly developed anti-inflammatory peptides (28). It remains to be determined whether this differential expression of NEMO in NK1R−/− will favor NF-κB over pathways modulated by AP-1.

Next, we searched for pathways downstream of NK1R activation using a novel statistically robust clustering method. We identified clusters of genes whose expression levels varied in a temporally coordinated manner. A network of highly correlated genes revealed a central place for NEP (M81591). Within the urinary tract, NEP is present in the urothelium, submucosa, and detrusor muscle (24, 41). Inhibition of NEP or mucosa removal potentiates peptide-induced bladder contraction (24, 41), and inflammation. In fact, the bladder and stomach of NEP knockout mice present spontaneous vascular leakage (23).

Fig. 6. Strongly correlated genes. *Time course variation in the ratio of gene expression (1, 4, and 24 h). Red boxes indicate upregulation LPS/saline > 3.0, and green boxes indicate downregulation saline/LPS > 3.0.

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WT mice. Possible modulators of NEP expression include hormones and inflammation. In regard to hormonal regulation of the NEP gene, it seems that this gene has at least two elements, including a typical androgen receptor element (ARE) which binds androgen, progesterone, and glucocorticoid receptors, and a unique androgen responsive region (ARR) which only binds androgen receptor (20). In terms of inflammation, the capacity of glucocorticoids to increase NEP gene expression and specific activity and downregulate SP receptors (17) may partly explain their anti-inflammatory properties. Our results are in agreement with findings that pathological conditions such as cancer (48), viral infection (39), and inflammation (16) markedly reduce NEP activity. Supporting our findings are the results indicating that recombinant NEP administration reduced bladder vascular response (46) and gastrointestinal inflammation (46). Our work adds to a long list of evidence that inflammation and infection can lead to NEP downregulation. These findings may have a serious impact on the recent evidence that NEP/neprilysin deficiency could be a cause or Alzheimer’s disease (20). The absence of downregulation of NEP in NK1R-/- suggests that either the inflammatory response itself or NK1R activation is upstream of NEP expression.

Another gene that switched to negative correlation was endoglin (CD105), a receptor for some of the members of the TGF-β family, which is expressed on proliferating endothelial cells (15). In the urinary tract, endoglin has been used as marker for survival in prostate cancer (58), and it was found upregulated in experimental renal fibrosis (36). In addition, two other genes, those encoding Mobp and lymphopain, switched from negative correlation in WT to a strongly positively correlation in NK1R-/- mice. Mobp (U81317) is an extracellular matrix myelin-associated oligodendrocytic basic protein (54). Lymphopain is another endopeptidase of the papain family, also named cathepsin W precursor (AF014941; Ref. 52). Finally, the majority of genes that exhibited temporally correlation did not present a differential expression between WT and NK1R-/- mice.

A network of highly correlated genes revealed two main clusters (Figs. 5 and 6). The first cluster had NEP in a central position highly correlated with a series of genes. NEP was strongly correlated with LAMA-3, TPA-11, and fos-B, which are known to be regulated by AP-1. The correlation of NEP with genes downstream of AP-1 may have an interesting consequence on cell survival during inflammation and cancer (34). Although the correlation between AP-1 and hormone-dependent gene expression is not clear, a downregulation of AP-1 activity induced by epithelial cell differentiation is a prerequisite to androgen-induced gene expression (9). In this context, the regulatory region of LAMA3 has three AP-1 bindings that are driven by Jun-Fos heterodimers (50). Moreover, Fos-B, a known AP-1 protein, can be induced by sensory C fiber stimulation that occurs in inflammation (33). In the same cluster, NEP was strongly correlated with genes such as TNF-β and LAMA-3 that are known to that share promoter elements. A potential link of laminin activity and peptidases is suggested by the finding that laminin fragments may have a regulatory role in cell migration (47).

Another interesting correlation was found between Cgrp receptor, Maf1, and TPA-11. CGRP is a sensory neuropeptide with regulatory on early B lymphocyte differentiation by a AP-1-dependent pathway (29). Maf belongs to a family of oncoproteins which encodes a nuclear basic Zip transcription factor protein. It has been reported that genes belonging to Maf family have critical roles in embryological development and cellular differentiation in the urinary tract, and Maf1 mRNA was localized in the kidney (18). Of relevance for this correlation, it was shown that Maf1 heterodimerizes with all four Fos proteins (27). Regarding TPA, electrophoretic mobility shift assays demonstrated the involvement of the AP-1 in the generation of plasminogen activator (25). Another positive correlation in the primary cluster was observed between the transcription factor Elf1 and TNF-β. This is in agreement with the analysis of TßR-II promoter/reporter gene constructs which demonstrates that two conserved Ets-binding sites play an important role in the activity of the TßR-II promoter (22). In addition to Maf1, Mnt was another zipper protein (30) identified in this cluster. In addition to zipper proteins, some of the other proteins of this cluster such NEP, SHAB (4), and CPR (21) share a common regulation by zinc.

The secondary cluster contains COL9A2, VHLH, MACIP, CPR, and PTP. The relationship between genes of the secondary cluster and the connection between the two clusters by the pairs NEP and COL9A2 and TGF-β and VHLH has been less documented. COL9A2 is an extracellular matrix protein present in kidney (44). Von Hippel Lindau (VHLH) is a tumor suppressor gene present in the kidney, and somatic mutations in VHLH occur in most clear cell renal carcinomas (11). PTP is a protein tyrosine phosphatase that plays a critical role in differentiation processes, particularly at their early stages (8), and MACIP is a mouse inhibitory G protein, transducins homolog, which play an essential role in NF-κB kinase (12). COL9A2 is an extracellular matrix protein present in kidney (44).

One of the most impressive contrasts in gene expression between WT and NK1R-/- was the switch of NEP correlation as demonstrated by the connectivity mosaic (Fig. 4) as well as by the network (Fig. 5). The mechanisms for this switch are not clear. However, it cannot be discarded that SP acting on NK1Rs may have a direct effect on NEP regulation. At least in fibroblast, addition of SP increases NEP mRNA (2). One possible explanation is that NEP is downstream of NK1R activation. Another possible explanation is that NEP is downstream of the inflammatory cascade. In conclusion, these results strongly indicate a key interregulation of NK1R and NEP genes which are known to be playing a key role in inflammation. It remains to be determined whether the same phenomenon would be
observed by pharmacological blocked of NK,R compared with gene deletion.

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